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Identification of Mutations in Chronic Lymphocytic Leukemia using ccf-DNA Isolated by Dielectrophoresis

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Manouchehri, Sareh

Publication Date
2015

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Identification of Mutations in Chronic Lymphocytic Leukemia using ccf-DNA Isolated by Dielectrophoresis

A thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Bioengineering

by

Sareh Manouchehri

Committee in charge:

Professor Michael Heller, Chair
Professor Pedro Cabrales
Professor Geert Schmid-Schoenbein

2015
The Thesis of Sareh Manouchehri is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2015
DEDICATION

to

My husband, my family and friends
“Yesterday I was clever, so I wanted to change the world. Today I am wise, so I am changing myself.”

-Rumi
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ACKNOWLEDGMENTS

This thesis would not have been possible without the guidance and the help of many individuals who helped me in completing my thesis.

First and foremost, I would like to thank Dr. Michael Heller for being an excellent mentor and teacher. His guidance and endless encouragements throughout my graduate school helped me to succeed in my research.

I would also like to thank my committee member Dr. Geert Schmid-Schoenbein, for being a great professor and always having his office door open for my questions. I am very grateful of my other committee member Dr. Pedro Cabrales who besides being a great mentor gave me the opportunity to assist him for his senior design class, from which I learned a lot.

My special thanks to Biological Dynamics for providing the equipment required for this research. I am especially thankful to Dr. Thomas Kipps and the members of his lab, Dr. Laura Rassenti, Dr. Emanuela Ghia, and Dr. George Widhopf II, for providing access to CLL samples.

I also must acknowledge all the people in Heller’s lab, especially Dr. Stuart Ibsen and Dr. Jennifer Wright, who besides being great friends, were my research mentors and always encouraged me throughout my journey. I also wish to thank Dr. Avery Sonnenberg, as he was the person who saw my interest in this field when I was
in my junior year, and taught me everything about DEP. I am also very thankful to my other friends in my research group: Dr. Augusta Modestino, Michelle Cheung, Elaine Skowronski, Sejung Kim, Taeseok Oh, Daniel Heineck. Graduate school would not have been such a wonderful and pleasant experience without them.

I would like to send my special thanks to my mother, father, brother, sister, and my in-laws who supported my decision to move across the world in perusing better life opportunities and always encouraged me to reach my dreams. Living so far from them is one of the hardest obstacles I had to deal with in my journey, yet I am glad I was able to make them proud.

Finally, I am especially dedicating my thesis to my dear husband, Arash. I would not be who I am today without his love and support and motivations.

The following co-authors have contributed immensely to the work presented in each chapter. I very much appreciate their help, support, and contributions.

**Chapter 1**, in part, is a reprint of the following manuscript: Rapid Electrokinetic Isolation of Cancer-Related Circulating Cell Free DNA Directly from Blood. Co-authors Avery Sonnenberg, Jennifer Marciniak, Laura Rassenti, Emanuela M. Ghia, Elaine Skowronski, Sareh Manouchehri, James McCanna, George F. Widhopf II, Thomas J. Kipps, Michael J. Heller. This has been published in Journal of Clinical Chemistry.

**Chapter 2**, in part, is a reprint of the following manuscript: Dielectrophoretic Isolation and Detection of Cancer Related Circulating Cell Free DNA Biomarkers from Blood and Plasma. Co-authors Avery Sonnenberg, Jennifer Marciniak, Elaine
Skowronski, Sareh Manouchehri, Laura Rassenti, Emanuela M. Ghia, George F. Widhopf II, Thomas J. Kipps, Michael J. Heller. This has been published in Journal of Electrophoresis.

**Chapter 3**, in part, is in preparation for submission for publication as: Dielectrophoretic Recovery of DNA from Plasma for the Identification of Chronic Lymphocytic Leukemia Point Mutations. Co-authors. Sareh Manouchehri Stuart Ibsen, Jennifer Wright, Laura Rassenti, Emanuela M. Ghia, George F. Widhopf II, Thomas J. Kipps, Michael J. Heller.
VITA

Master of Science, Bioengineering; December 2015
University of California San Diego, La Jolla, CA

Bachelor of Science, Bioengineering; June 2013
University of California San Diego, La Jolla, CA

PUBLICATIONS

Sareh Manouchehri, Stuart Ibsen, Jennifer Wright, Laura Rassenti, Emanuela M. Ghia, George F. Widhopf II, Thomas J. Kipps and Michael J. Heller. Dielectrophoretic Recovery of DNA from Plasma for the Identification of Chronic Lymphocytic Leukemia Point Mutations, in submission

Stuart Ibsen, Jennifer Wright, Se Jung Kim, Seo-Yeon Ko, Jiye Ong, Sareh Manouchehri, Johnny Akers, Clark Chen, Michael Heller. Recovery of Exosomes Containing Circulating Cell-Free RNA from Human Plasma Using High Conductance Dielectrophoresis. in submission


FIELDS OF STUDY

Major Field: Bioengineering

Professor Michael J. Heller
ABSTRACT OF THE THESIS

Identification of Mutations in Chronic Lymphocytic Leukemia using ccf-DNA Isolated by Dielectrophoresis

by

Sareh Manouchehri

Master of Science in Bioengineering

University of California, San Diego, 2015

Professor Michael Heller, Chair

Circulating cell-free (ccf) DNA has become an important biomarker for the early detection, monitoring, and treatment of cancers. However, the current gold standard methods of isolating ccf-DNA from blood or plasma are labor intensive and time consuming. The complex sample processing procedures may cause degradation or loss of ccf-DNA, and thus greatly inhibit the use of ccf-DNA as a target biomarker for point of care (POC) diagnostics. Therefore, it is essential to develop a rapid and inexpensive ccf-DNA extraction method in order to use ccf-DNA as a biomarker for clinical applications.
The work described in this thesis demonstrates a dielectrophoretic- (DEP) based method for the rapid isolation of ccf-DNA from undiluted whole blood and plasma samples collected from chronic lymphocytic leukemia (CLL) patients. It is demonstrated that DEP can recover ccf-DNA from 25 µL of blood and plasma in less than 15 minutes. To investigate the potential of DEP for use in clinical applications, the dielectrophoretically recovered ccf-DNA from blood and plasma of CLL patients was amplified using polymerase chain reaction (PCR) and sequenced for CLL specific mutations. The results of the genetic analysis were found to be comparable to sequencing results obtained from ccf-DNA isolated by conventional golden standard methods as well as the DNA extracted directly from B-cells. The ability of DEP-based technology to rapidly isolate ccf-DNA from small volumes of unprocessed blood shows the potential to accelerate sample processing and enable the use of ccf-DNA as a specific biomarker target for point of care diagnostics.
INTRODUCTION

Circulating Cell-Free (ccf) DNA as a Biomarker

Cancer is one of the most common causes of death worldwide. Over the past few decades, many studies have been looking to define a cancer biomarker that will provide patient specific information for accurate disease diagnosis and provide information to increase the efficacy of treatment. Most cancer biomarkers discovered so far cannot be used for routine clinical applications due to a lack of sensitivity [1]. Also, in the case of solid tumors, many procedures require invasive biopsies to obtain the necessary information [1]. Cancer can start with random somatic gene mutations caused as a consequence of natural of aging and environmental factors all causing DNA damage. Information about these mutations can help predict the prognostics of the disease and find the appropriate treatment method [2]. Recently, much effort has been made in finding a non-invasive biomarker that can be used for diagnostic and treatment monitoring to simplify cancer management [3].

Cell free circulating (ccf) DNA is one of the biomarkers that recently has got a lot of attention [3]. The existence of cell free DNA in the plasma was first discovered by Mandel and Metais in 1948 [4] but its correlation with cancer was not discovered until 1977 by Leon et al.[5]. Ccf-DNA circulates in the blood of healthy people at a certain background level (<50 ng/mL). This could be due to routine cell housekeeping functions, such as the process of macrophages and the phagocytes which degrade genomic DNA into small apoptotic fragments. Ccf-DNA can also originate from cancerous cells that release it into the blood due to widespread necrosis and apoptosis in the tumor. It has been shown that ccf-DNA levels elevate in various types of cancer,
and often exhibit the same genetic alterations as DNA isolated from related cancer cells and tumor tissues. Thus, the ability to use ccf-DNA as a cancer biomarker creates a form of ‘liquid biopsy’ with the potential to replace the surgically invasive tumor biopsy in the future. Ccf-DNA can provide specific targets for diagnosis as well as permitting treatment monitoring as it exists at all stages of the disease [3, 6].

Currently, the process and the techniques of recovery of ccf-DNA are complex, and labor and time intensive. They usually require more than 1 mL of plasma to start, and also include an extended amount of time and labor between every step from the initial blood draw and cell separation to the final DNA extraction. This could cause degradation of the sample due to mechanical shearing or lead to loss of useful material during the sample preparation procedures. Therefore, these sample preparation complications greatly inhibit the possibility of using ccf-DNA for point of care (POC) diagnostics [3, 6]. Therefore, it is essential to develop a rapid and inexpensive isolation method in order to use ccf-DNA as a biomarker for clinical applications.

**Dielectrophoresis**

Electrokinetic-based methods such as DC electrophoresis and dielectrophoresis have been used for biological applications and particle separations [3, 7]. One example is the use of gel electrophoresis for DNA size separation. Dielectrophoresis is an electrokinetic technique which uses an alternating electric field to create a separation force based on differences between the dielectric properties of the particle and its surrounding medium [8]. When a dielectric particle, such as a particle made of DNA, is placed in an electrical field, it will become polarized and generate a dipole moment. This dipole moment is frequency dependent and its magnitude depends on the
polarizability of the material in the particle relative to the surrounding media. In a non-uniform alternating current (AC) electric field these particles will experience a DEP force that moves them into the high or low field region. A particle with a higher polarizability than its suspending medium will experience a positive DEP force which pushes it towards the region with higher electric field strength (high field) and the particle with lower polarizability compared to its medium, will be driven to the area with lower electric field strength (low field) by the force of negative DEP [9]. Using an appropriately designed microelectrode device, these dielectric particles can be concentrated into areas of high or low electric field. This thesis demonstrates that ccf-DNA can be rapidly isolated using this DEP technique from the rest of the particles in whole plasma or blood.

References


Thesis Organization

In this thesis the ability of dielectrophoresis to recover ccf-DNA will be investigated. This thesis is organized into three chapters.

Chapter 1 – In this chapter the ability of DEP to isolate ccf-DNA from undiluted whole blood of Chronic Lymphocytic leukemia (CLL) patients is investigated. We performed genetic analysis on the recovered DNA and found it to be comparable to results obtained using DNA extracted using golden standard methods. This has been published in the Journal Clinical Chemistry.

Chapter 2 – In this chapter the ability of DEP to recover the ccf-DNA from both plasma and the blood of CLL patients will be investigated. The genetic information obtained from the recovered ccf-DNA was compared with information obtained from genomic DNA. This work has been published in the journal of Electrophoresis.

Chapter 3 – In this chapter, the dielectrophoretic recovered ccf-DNA from archived CLL plasma samples was analyzed to identify point mutations. The mutation status of DEP recovered DNA was compared to the point mutations found in the DNA isolated from B-cells of the same CLL patients. This work is in preparation for submission to the International Journal of Hematologic Oncology.
CHAPTER 1
Rapid Electrokinetic Isolation of Cancer-Related Circulating Cell Free DNA
Directly from Blood

1.1 INTRODUCTION

Circulating cell-free (ccf) DNA is now considered an important biomarker for early
detection of cancer [1-4], residual disease [5], monitoring chemotherapy [6] and other
aspects of cancer management [1, 7-13]. The isolation of ccf-DNA from plasma as a “liquid
biopsy” will begin to replace more invasive tissue biopsies as a means to detect and analyze
cancer mutations [1, 7, 9-12]. Unfortunately, conventional methods and techniques for the
isolation of ccf-DNA from plasma are extremely time-consuming and complex processes.
These are major drawbacks that greatly limit many biomedical research applications, and
rule out the use of ccf-DNA biomarkers for point-of-care (POC) diagnostic applications.
Other limitations of these conventional sample preparation methods and processes include:
(1) the procedures generally require starting with at least one or more milliliters of plasma;
(2) obtaining the plasma from blood requires centrifugation and pipetting steps; (3) the
large number of manipulations increases the chance for technician errors; (4) the extended
time and multiple processing steps add considerable cost to the diagnostic test; (5) the ccf-
DNA recovery efficiency decreases as sample size decreases and as the concentration of
ccf-DNA in the sample decreases; (6) ccf-DNA can be degraded by mechanical sheering
during the many processing steps; and (7) the degradation and loss of higher
nanoparticulate forms of ccf-DNA limit our knowledge of its true in vivo nature.
In the case of hematological cancers such as chronic lymphocytic leukemia (CLL) and lymphomas, DNA can be obtained from the transformed cancer cells [14, 15] or from isolation of ccf-DNA from plasma [16]. The B cells of CLL patients can be segregated into one of at least two major subsets on the basis of whether or not the immunoglobulin (IG) variable region has somatic mutations [17]. Patients with CLL cells that express unmutated IG heavy chain variable region genes (IgVH genes) tend to have an aggressive clinical course relative to that of patients who have CLL cells that express IgVH with somatic mutations [18-20]. For CLL diagnostics and management, DNA is isolated from the peripheral blood mononuclear cells (PBMCs). The PBMCs are usually purified from the CLL patient blood samples by density centrifugation using Ficoll-Hypaque 1077. This is a long and labor-intensive process, which adds considerable cost to patient management. PCR and DNA sequencing are performed on the isolated B-cell DNA to determine the mutation status for the expressed IgVH gene [21-23].

Promising electrokinetic technologies, in particular dielectrophoresis (DEP) have long been known to provide effective separation of cells, nanoparticles, DNA and other biomolecules [24-26]. Until recently, DEP techniques remained impractical for general use with high-conductance solutions (5-15 mS/cm), which include important clinical samples such as whole blood, plasma and serum [25, 26]. In earlier work, sample dilution to low-conductance conditions (<1 mS/cm) was required before effective DEP separations could be carried out [26-29]. While some progress was made in using DEP under high-conductance conditions, these efforts have been limited to separations of cells and micron-sized entities by negative DEP forces using hybrid electrokinetic devices [27, 30-32]. The
devices still could not be used with whole blood samples and, more importantly, did not provide isolation of DNA from the sample. We have developed an electrokinetic technique that allows nanoscale entities, including high molecular weight (hmw) DNA and nanoparticles, to be isolated from high-conductance (>10 mS/cm) solutions [33-35], whole blood samples [36], and ccf-DNA from blood samples [37]. In this study, we show fluorescent analysis, PCR and Sanger sequencing results for ccf-DNA isolated by DEP from 25 µL samples of unprocessed CLL patient blood. PCR and Sanger sequencing results for the DEP process are compared to results obtained using conventional sample preparation of ccf-DNA from 1 mL of CLL patient plasma, and to DNA sequencing results obtained directly from leukemic B cells. The ability to rapidly isolate ccf-DNA, RNA and other nanoparticulate biomarkers directly from blood in their \textit{in vivo} forms will provide an advantage to basic biomedical research to expedite discoveries and treatments for a variety of diseases.

1.2 MATERIALS and METHODS

1.2.1 Sample Acquisition

Blood samples were collected from fifteen CLL patients and three healthy volunteers (IRB#: 080918) in collection tubes containing lithium heparin (BD). For the dielectrophoresis experiments, 300 µL of blood was taken from the top of each undisturbed blood sample within 4-5 hours of collection. The remaining blood was then centrifuged for 10 minutes at 1100 RCF and the supernatant (plasma) was pipetted into a microcentrifuge tube.
1.2.2 Qiagen DNA Extraction from Plasma

The QIAamp Circulating Nucleic Acid kit was used to extract ccf-DNA from 1 mL of plasma from each of the CLL patients and healthy donors. After addition of a lysing buffer and 30 minutes of incubation, the plasma mixture was pulled through a silica binding column with a vacuum manifold, followed by three washing steps on the vacuum manifold. After a 10-minute incubation at 56 °C to dry the membrane, the DNA was eluted into the provided elution buffer by centrifugation for 1 minute at 20,000 RCF and stored at 4 °C.

1.2.3 DNA Extraction on AC Electrokinetic Microarray

New AC electrokinetic microarray devices (Biological Dynamics, La Jolla, CA) allow the rapid isolation of ccf-DNA directly from a small volume of unprocessed blood. Figure 1A shows the 10 mm x 20 mm microarray device containing 1000 microelectrodes, each 60 µm in diameter. The expanded view shows a section of the chip which is fabricated on a silicon base with platinum microelectrodes insulated by SiO₂ and over-coated with a porous hydrogel layer (Poly-2-hydroxyethyl methacrylate). The expanded view also shows the location of the DEP high-field regions (green) and the DEP low-field regions (red) which form when the AC field is applied.

In the first step of the process, a blood sample containing the ccf-DNA is placed into the microarray device and an AC electric field is then applied (Figure 1.1 B). At a specific AC frequency and voltage, the ccf-DNA, which is more polarizable than the surrounding media, experiences positive DEP (p-DEP), which causes it to concentrate into the DEP high-field regions over the circular microelectrode structures. The blood cells,
which are less polarizable, experience negative DEP (n-DEP), which causes them to move into the DEP low-field regions between the microelectrodes (Figure 1.1C). Concentration of the ccf-DNA into the DEP high-field regions requires only three minutes, after which a fluid wash removes the blood cells and other blood components from the microarray (Figure 1.1D). After the washing step the ccf-DNA, if fluorescently stained, can be analyzed on-chip by fluorescence microscopy and the sample can be eluted for subsequent PCR and DNA sequencing analysis.
Each microarray device (chip) was pre-treated by adding 25 µL of 0.5x PBS (Lonza) to the flow cell and applying a 2 V<sub>RMS</sub>, 5 Hz sinusoidal waveform for 15 seconds to improve the hydrogel porosity. The 0.5x PBS was then removed and 25 µL of blood was added to the flow cell. An 11 Volt peak-to-peak (V<sub>p-p</sub>), 10 kHz sinusoidal waveform was then applied to the chip for 3 minutes with no fluid flow. The same electric field was maintained while the chip was washed for 5 minutes at 200 µL/min with 1x TE (Sigma-
Aldrich). The electric field was then turned off, allowing captured DNA to diffuse into the 1x TE solution. The 25 µL of fluid was removed within 30 seconds and stored in a microcentrifuge tube. For each CLL patient and healthy donor, this process was repeated 4 times, each time on a new microelectrode device. The 25 µL of eluted sample from each of the 4 runs was combined into a single microcentrifuge tube (100 µL total volume) and stored at 4°C.

In order to visualize collection on the microelectrode array, the CLL and healthy donor samples were stained with SYBR Green I fluorescent double-stranded DNA dye (Life Technologies, Carlsbad, CA). One and a half microliters of 100x SYBR Green I was added to 28.5 µL of blood and allowed to incubate at room temperature for 5 minutes. 25 µL of this solution was added to the device and run as described above. After the 3 minutes of electric field collection and 5 minutes of washing, bright field and fluorescent images of the microelectrode pads were acquired using a CCD camera with a 10x objective, FITC filter, and a 470 nm LED excitation source. DNA with SYBR Green I from these imaged devices was not eluted or used in subsequent analysis.

1.2.4 DNA Quantification

The DNA collected using both the DEP and Qiagen protocols was quantified using Quanit-iT PicoGreen (Life Technologies), a double-stranded DNA dye. Each sample was diluted and combined with the PicoGreen reagent, and the resulting fluorescence was measured with a fluorescence plate reader (Tecan).
1.2.5 Gel Electrophoresis, PCR Analysis, and Sequencing.

In order to verify that the collected DNA was from leukemic B cells, it was amplified by PCR using Phusion High-Fidelity DNA Polymerase (New England Biolabs). The forward primers used were specific to the VH1, VH3, and VH4 regions, and the reverse primer was specific for the JH region. PCR thermal cycling conditions were a 5-minute initial denaturation at 98 °C followed by 40 cycles of 98 °C denaturation for 15 seconds, 66 °C annealing for 15 seconds, and 72 °C extension for 15 seconds. The PCR product was analyzed by gel electrophoresis on a 2% agarose gel containing ethidium bromide (Life Technologies). The gels were viewed in a transilluminator and images were captured using a CCD camera. The images were analyzed with ImageJ software to determine the fluorescence in the region where the main 500-550 bp CLL target fragments should appear, regardless of whether or not a discrete band was observed. Remaining PCR product was cleaned with the QIAquick PCR purification kit (Qiagen) and sequenced by Sanger sequencing.

1.2.6 B-cell Isolation and IgVH Analysis

Peripheral blood mononuclear cells were isolated by density centrifugation using Ficoll-Hypaque 1077 (Sigma-Aldrich, St. Louis, MO) and suspended in fetal-calf serum containing 10% dimethylsulfoxide (Sigma–Aldrich) for storage in liquid nitrogen. DNA was extracted using the QIAamp DNA Mini kit (Qiagen, Valencia, CA) and eluted in 30 mL of nuclease-free water. IgVH gene characterization and mutation status was assessed as previously described [38]. Most PCR products were sequenced directly, although in
some cases, amplified products were cloned into pGEM-T (Promega, Madison, WI). Nucleotide sequences were analyzed using the ImMunoGenetic (IMGT) directory (European bioinformatics Institute ImMunoGeneTics Informations System available at http://imgt.cines.fr) (Leukemia 2011 Langerak, Davi, ERIC guidelines) [22]. Sequences with less than 98% homology with the corresponding germline IgVH gene were considered mutated. The heavy chain complementarity-determining region (HCDR3) was determined [23] as defined by the number of amino acids between codon 94 at the end of framework 3 and the conserved Trp of position 102 at the beginning of framework 4.

1.3 RESULTS

1.3.1 ccf-DNA Isolation from CLL Samples

In this study, AC electrokinetic microarrays were used to isolate ccf-DNA from fifteen CLL patient blood samples and three normal blood samples. The study shows for the first time that an on-chip dielectrophoresis technique allows: (1) isolation of ccf-DNA directly from unprocessed blood, (2) on-chip fluorescence analysis of ccf-DNA in under five minutes, and (3) elution of ccf-DNA for subsequent analysis in under ten minutes. The manipulations for the DEP process comprise two simple steps: the addition of the blood sample into the microarray device and removal of the eluted sample upon completion of the process. To compare the DEP process to a conventional ccf-DNA sample preparation method, the Qiagen QIAamp Circulating Nucleic Acid procedure was used to isolate ccf-DNA from plasma from the same fifteen CLL patients and three normal individuals. The Qiagen procedures are frequently used for isolation of ccf-DNA from cancer patient plasma.
samples [7, 10, 13]. Sanger sequencing results for ccf-DNA isolated with both the DEP and Qiagen procedures were compared to those obtained from isolated leukemic B cells. Figure 2 shows a comparison of the processing time and number of manipulations required for the DEP procedure (A), the Qiagen QIAamp Circulating Nucleic Acid procedure (B), and the CLL Lab procedure (C). The processing times for the Qiagen procedure and the CLL Lab procedure include only the actual time necessary to run a specific processing step (i.e. 10 minutes for centrifugation). They do not include the time necessary for setup, carrying out transfers such as pipetting, and other manipulations. When these processes are performed manually the additional manipulations can add an hour or more to the total time required for the Qiagen and CLL Lab processes.
1.3.2 On-Chip Fluorescence Detection of ccf-DNA

For on-chip fluorescence detection of the ccf-DNA, SYBR® Green I (Invitrogen) stain is added to the blood samples before the application of the DEP field. After DEP is carried out for three minutes and blood cells are removed by a fluidic wash, the fluorescently stained ccf-DNA, which is concentrated in the DEP high-field regions (on the microelectrodes), is detected. Figure 1.3 shows the fluorescent image results for ccf-
DNA isolated by DEP from one normal blood sample (Normal-1) and two representative CLL blood samples (CLL-9, CLL-10). On the far right are 3D fluorescence intensity images, which allow visualization of the relative amounts of ccf-DNA that were isolated. Overall, the fluorescent DNA levels were higher in most of the CLL patient samples when compared to the fluorescent DNA levels obtained from the normal blood samples.

Figure 1.3 – Fluorescence detection of ccf-DNA in CLL patient and normal blood samples.
On-chip fluorescence imaging results from 25 µL blood samples showing SYBR Green stained ccf-DNA that was concentrated on the edge of the electrodes. Images of one normal blood sample (Normal-1) and two representatives CLL blood samples (CLL-9 and CLL-10). Yellow dotted square areas in the images on the left side are enlarged in the center column images. The right side column shows 3D fluorescence intensity images created by MATLAB, which provide better visualization of the relative amounts of ccf-DNA that were isolated on the DEP high-field areas over the microelectrodes.
1.3.3 DNA Concentration in the Eluted Samples

Quant-iT PicoGreen (Invitrogen) fluorescence analysis was used to determine the concentration of ccf-DNA in samples eluted from the AC electokineic microarray and the Qiagen QIAamp Circulating Nucleic Acid procedure. For these experiments, SYBR Green I DNA dye was not added to the blood samples prior to DEP. Figure 1.4 shows the ccf-DNA concentration results for the eluted samples starting with 25 µL of blood for the DEP process (red bars) and 1 mL of plasma for the Qiagen process (blue bars). No correlation could be found between the DEP blood results and Qiagen plasma results for the CLL samples. For the CLL samples (n=15), the average (± standard deviation) concentration of DNA isolated by the DEP process (557 ± 450 ng/mL) was very similar to (p = 0.73) the Qiagen process (502 ± 436 ng/mL). For the normal samples (n=3), the average concentration of DNA isolated by the DEP process (162 ± 97.6 ng/mL) was higher, though not significantly (p = 0.13), than the Qiagen process (50.3 ± 25.5 ng/mL).

The eluted samples for both the DEP process and the Qiagen QIAamp Circulating Nucleic Acid process were amplified using primers for the IgVH1, IgVH3, and IgVH4 regions. For the DEP process, eluted ccf-DNA from the equivalent of 5 µL of the original CLL blood sample was amplified. For the Qiagen process, eluted ccf-DNA from the equivalent of 100 µL of the original 1 mL plasma samples was amplified. Table 1.1 compares the IgVH PCR band intensity results for ccf-DNA isolated from blood using the DEP process with the results obtained for ccf-DNA isolated using the Qiagen process. In Table 1.1, the PCR product band intensities for correct IgVH type (IgVH1, IgVH3, IgVH4) are in bold, and secondary IgVH bands, which have intensities more than 20% of those for
the correct IgVH type, are italicized. The correct IgVH PCR amplification products were obtained for ccf-DNA from all fifteen CLL patient samples using both the DEP process and the Qiagen process. Both the DEP and Qiagen processes also produced secondary IgVH PCR bands; nine bands for DEP and nine bands for Qiagen.

Figure 1.4 – Concentration of ccf-DNA in the DEP and Qiagen eluted samples. 
Bar graph of the ccf-DNA concentrations in the final eluted samples that were obtained directly from blood using the DEP process (black bars), and of the final eluted samples that were obtained from plasma using the Qiagen process (purple bars). The DNA concentrations were determined by fluorescence analysis using Quant-iT PicoGreen (Invitrogen) assay for double-stranded (ds) DNA and normalized to the original sample volume.
Table 1.1 – IgVH PCR band intensities for DEP blood and Qiagen plasma.

Highlighted results are IgVH (1, 3, or 4) which correlate with the previously obtained data from the CLL Lab.

<table>
<thead>
<tr>
<th>Patient</th>
<th>DEP</th>
<th>Qiagen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VH1</td>
<td>VH3</td>
</tr>
<tr>
<td>CLL - 1</td>
<td>2,110</td>
<td>2,520</td>
</tr>
<tr>
<td>CLL - 2</td>
<td>45,200</td>
<td>2,170</td>
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<tr>
<td>CLL - 3</td>
<td>78,900</td>
<td>1,780</td>
</tr>
<tr>
<td>CLL - 4</td>
<td>62,000</td>
<td>37,400</td>
</tr>
<tr>
<td>CLL - 5</td>
<td>1,440</td>
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</tr>
<tr>
<td>CLL - 6</td>
<td>1,930</td>
<td>60,500</td>
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<tr>
<td>CLL - 7</td>
<td>55,100</td>
<td>5,150</td>
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<tr>
<td>CLL - 8</td>
<td>54,500</td>
<td>1,860</td>
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<tr>
<td>CLL - 9</td>
<td>1,880</td>
<td>59,800</td>
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<tr>
<td>CLL - 12</td>
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<td>CLL - 13</td>
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<td>9,240</td>
</tr>
<tr>
<td>CLL - 14</td>
<td>1,900</td>
<td>14,500</td>
</tr>
<tr>
<td>CLL - 15</td>
<td>1,920</td>
<td>23,600</td>
</tr>
</tbody>
</table>

1.3.4 DNA Sequencing Results

Once the IgVH regions for each of the CLL samples were analyzed using PCR, the resulting PCR products were sequenced (Sanger sequencing) and compared to those obtained from isolated B-cells. This step verified that the isolated ccf-DNA came from the leukemia cell population. The results in Table 1.2 show that for all fifteen CLL samples, the sequences from the ccf-DNA isolated by DEP and by the Qiagen process matched those from isolated B-cells.
Table 1.2 – DNA sequencing results.

DNA sequencing results for ccf-DNA isolated from CLL blood samples by DEP and ccf-DNA isolated from CLL patient plasma samples by Qiagen process compared with the results obtained using an established method performed on genomic DNA obtained from CLL patient leukemic cells.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>DEP Blood</th>
<th>V-Gene/Allele</th>
<th>V-Region ID %</th>
<th>Qiagen Plasma</th>
<th>V-Gene/Allele</th>
<th>V-Region ID %</th>
<th>Genomic DNA</th>
<th>V-Gene/Allele</th>
<th>V-Region ID %</th>
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<tbody>
<tr>
<td>CLL – 1</td>
<td>IGHV4-39*01 F</td>
<td>95.53%</td>
<td>IGHV4-39*01 F</td>
<td>95.53%</td>
<td>IGHV4-39*01 F</td>
<td>95.53%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLL – 2</td>
<td>IGHV1-69*01 F</td>
<td>100.00%</td>
<td>IGHV1-69*01 F</td>
<td>100.00%</td>
<td>IGHV1-69*01 F</td>
<td>100.00%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLL – 3</td>
<td>IGHV3-30*03 F</td>
<td>95.14%</td>
<td>IGHV3-30*03 F</td>
<td>95.14%</td>
<td>IGHV3-30*03 F</td>
<td>95.14%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLL – 4</td>
<td>IGHV1-2*02 F</td>
<td>100.00%</td>
<td>IGHV1-2*02 F</td>
<td>100.00%</td>
<td>IGHV1-2*02 F</td>
<td>100.00%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLL – 5</td>
<td>IGHV4-4*07 F</td>
<td>100.00%</td>
<td>IGHV4-4*07 F</td>
<td>100.00%</td>
<td>IGHV4-4*07 F</td>
<td>100.00%</td>
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<td></td>
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<tr>
<td>CLL – 6</td>
<td>IGHV1-8*01 F</td>
<td>100.00%</td>
<td>IGHV1-8*01 F</td>
<td>100.00%</td>
<td>IGHV1-8*01 F</td>
<td>100.00%</td>
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<td></td>
</tr>
<tr>
<td>CLL – 7</td>
<td>IGHV3-21*02 F</td>
<td>88.19%</td>
<td>IGHV3-21*02 F</td>
<td>88.19%</td>
<td>IGHV3-21*02 F</td>
<td>88.19%</td>
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<tr>
<td>CLL – 8</td>
<td>IGHV3-33*01 F</td>
<td>94.10%</td>
<td>IGHV3-33*01 F</td>
<td>94.10%</td>
<td>IGHV3-33*01 F</td>
<td>94.10%</td>
<td></td>
<td></td>
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<tr>
<td>CLL – 9</td>
<td>IGHV1-69*01 F</td>
<td>100.00%</td>
<td>IGHV1-69*01 F</td>
<td>100.00%</td>
<td>IGHV1-69*01 F</td>
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<td>CLL – 10</td>
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<td>97.19%</td>
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</tr>
<tr>
<td>CLL – 11</td>
<td>IGHV3-64*01 F</td>
<td>94.79%</td>
<td>IGHV3-64*01 F</td>
<td>94.79%</td>
<td>IGHV3-64*01 F</td>
<td>94.79%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLL – 12</td>
<td>IGHV3-48*03 F</td>
<td>98.96%</td>
<td>IGHV3-48*03 F</td>
<td>98.96%</td>
<td>IGHV3-48*03 F</td>
<td>98.96%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLL – 13</td>
<td>IGHV4-39*01 F</td>
<td>96.91%</td>
<td>IGHV4-39*01 F</td>
<td>96.91%</td>
<td>IGHV4-39*01 F</td>
<td>96.91%</td>
<td></td>
<td></td>
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<tr>
<td>CLL – 14</td>
<td>IGHV4-34*01 F</td>
<td>99.30%</td>
<td>IGHV4-34*01 F</td>
<td>99.30%</td>
<td>IGHV4-34*01 F</td>
<td>99.30%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLL – 15</td>
<td>IGHV4-39*01 F</td>
<td>100.00%</td>
<td>IGHV4-39*01 F</td>
<td>100.00%</td>
<td>IGHV4-39*01 F</td>
<td>100.00%</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
1.4 DISCUSSION

Ccf-DNA and ccf-RNA have the potential to become important biomarkers for diagnostics and patient management in almost all hematological and solid tumor cancers. Ccf-DNA/RNA isolated from plasma, constituting a “liquid biopsy,” may serve as an alternative to more invasive tissue biopsies in the detection and analysis of cancer mutations. Unfortunately, the time, complexity, and cost of employing conventional methods to isolate ccf-DNA/RNA from plasma can limit the use of these procedures, especially for point-of-care diagnostic applications. This study demonstrates the ability of an on-chip dielectrophoresis technique to isolate ccf-DNA directly from unprocessed blood. The DEP process comprises only two steps and can be completed in less than 10 minutes from 25 μL of blood. In contrast, the Qiagen procedure typically involves obtaining 1-2 mL of plasma from 2-3 mL of blood and subsequently subjecting the plasma to a series of manipulations to obtain ccf-DNA over the course of 1-2 hours. The present process for isolating genomic DNA from B-lymphocytes requires 15-20 mL of blood and takes several hours to complete. The DEP process enables the use of unprocessed blood samples and reduces the cost and complexity of ccf-DNA isolation relative to the Qiagen and standard CLL Lab protocols, while providing comparable PCR and DNA sequencing results.

An additional advantage of using the DEP process is the ability to carry out fluorescence detection of ccf-DNA within minutes of application of the blood sample to the chip. The use of fluorescence to rapidly determine ccf-DNA levels in clinical blood samples could ultimately provide a first level “alarm” for POC diagnostics. In the case of solid tumors, researchers have demonstrated a correlation between ccf-DNA levels in patient plasma and survivability for lung and colon cancers [39, 40]. However, the isolation of ccf-DNA in these
studies required long and involved processes. We have previously demonstrated rapid semi-quantitative fluorescence detection results for the DEP isolation of DNA spiked into serum that spanned 8 ng/mL to 500 ng/mL, which would be a useful dynamic range for measuring ccf-DNA in clinical samples [37].

In addition to on-chip fluorescence analysis, the concentrations of ccf-DNA from the DEP process and the Qiagen process were determined by fluorescence after elution. Both the DEP and Qiagen methods showed, on average, higher ccf-DNA concentration levels for CLL patients than for normal patients. In many cases the ccf-DNA levels of CLL samples were substantially higher compared to those of normal samples. However, we did not detect a correlation between the CLL sample ccf-DNA levels obtained by the DEP and Qiagen methods for each patient. This lack of correlation may be due, at least in part, to the fact that the DEP method isolates DNA directly from blood while the Qiagen process uses plasma. It is very likely that the ccf-DNA isolated directly from blood is more representative of the actual in vivo ccf-DNA size range than is ccf-DNA isolated from the plasma. The numerous processing steps required for the extraction of ccf-DNA from plasma cause shearing and degradation of higher molecular weight ccf-DNA into small fragments.

The primary goal of this study was achieved in demonstrating that the PCR and DNA sequencing results obtained by the DEP process were comparable to those obtained by the Qiagen and CLL Lab processes. The IgVH analysis revealed that in all fifteen CLL patients, the IgVH sequencing results obtained using ccf-DNA isolated by both DEP and the Qiagen process matched exactly the original IgVH patient-specific sequencing results obtained from B-cell DNA. To the best of our knowledge, this work represents the first study of CLL or any other cancer carried out using ccf-DNA isolated directly from an unprocessed blood sample.
In summary, the DEP technique shows potential for enabling rapid, simple and cost-effective “liquid biopsy” and POC cancer diagnostics. In addition, the DEP technique may become a useful tool for biomedical research. Currently, the true in vivo nature and actual levels of ccf-DNA/RNA, exosomes and other nanoparticulate biomarkers in blood are not well known. The ability to rapidly isolate, in their unperturbed states, the cellular nanoparticulates released into the bloodstream by injured, necrotic and transformed cells is critical to a better understanding of the disease process itself. Unquestionably, conventional sample preparation procedures, which involve processing plasma from blood and subsequently subjecting plasma to numerous time-consuming/labor-intensive physical manipulations, may lead to loss and degradation of the biomarkers. The use of this DEP technique for rapid isolation of ccf-DNA/RNA directly from blood samples may also provide biomarkers in their unperturbed state, enabling improved biomarker isolation for research and better diagnostic tools.

1.5 ACKNOWLEDGMENTS

Chapter 1, in part, is a reprint of the following manuscript: Rapid Electrokinetic Isolation of Cancer-Related Circulating Cell Free DNA Directly from Blood. Co-authors Avery Sonnenberg, Jennifer Marciniak, Laura Rassenti, Emanuela M. Ghia, Elaine Skowronsksi, Sareh Manouchehri, James McCanna, George F. Widhopf II, Thomas J. Kipps, Michael J. Heller. This has been published in Journal of Clinical Chemistry.
1.6 REFERENCES


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CHAPTER 2
Dielectrophoretic Isolation and Detection of Cancer Related Circulating Cell Free DNA Biomarkers from Blood and Plasma

2.1 INTRODUCTION

Circulating cell-free (ccf) DNA is an important biomarker for early detection of cancer [1-4], residual disease [5, 6], monitoring chemotherapy [7] and other aspects of cancer management [1, 8-15]. The isolation of cancer-related ccf-DNA from plasma may allow “liquid biopsies” to replace more invasive tissue biopsies for detecting and analyzing cancer mutations [1, 8, 10-14, 16-19]. However, the present methods for isolating ccf-DNA from plasma are complex, time-consuming and relatively expensive processes that rule out use for point-of-care (POC) diagnostic applications. Conventional sample preparation processes have many other limitations, which include: (1) requirement of at least one or more milliliters of plasma; (2) the processing of blood to plasma; (3) a large number of manipulations, which increases the chance for technician errors; (4) decrease of recovery efficiency with decrease in sample size and concentration; (5) degradation of ccf-DNA by mechanical sheering during the processing steps; and (6) limiting PCR analysis to shorter target DNA sequences due to the degradation of ccf-DNA. Finally, other potentially important cancer-related biomarkers such as ccf-RNA, exosomes and microvesicles also require relatively long and involved processes for their isolation from plasma. With regard to hematological cancers such as chronic lymphocytic leukemia (CLL) and lymphomas, DNA for PCR and sequencing can be obtained from transformed cells [20, 21], as well as
from ccf-DNA isolated from plasma [22]. In the case of CLL, B-cells from patients can be segregated into one of at least two major subsets on the basis of whether or not the immunoglobulin (Ig) variable region has somatic mutations [23]. Patients with CLL cells that express unmutated Ig heavy chain variable region genes (IgVH genes) tend to have an aggressive clinical course relative to that of patients who have CLL cells that express IgVH with somatic mutations [24-26]. For CLL diagnostics and management, genomic DNA is isolated from the peripheral blood mononuclear cells (PBMCs).

The PBMCs are usually purified from the CLL patient blood samples by density centrifugation using Ficoll-Hypaque 1077. This is a long and labor-intensive process, which adds considerable cost to patient management and precludes any POC applications. To assess the unique patient-specific IgVH expressed by the CLL B-cells, PCR and DNA sequencing are performed on the isolated genomic DNA to determine the mutation status for the expressed IgVH gene [27-29].

Electrokinetic technologies, like AC dielectrophoresis (DEP) have long been known to provide effective separation of cells, nanoparticles, DNA and other biomolecules [30-36]. However, until recently, DEP techniques remained impractical for use with high-conductance solutions (5-15 mS/cm), as well as with whole blood, plasma and serum [33-36]. In earlier work, sample dilution to low-conductance conditions (<1 mS/cm) was required before effective DEP separations could be carried out [32, 35-49]. While some progress was made using DEP under high-conductance conditions, these efforts have been limited to separations of cells and micron-sized entities by negative DEP forces using hybrid electrokinetic devices [37, 50-54]. Such devices still could not be used with whole
blood samples, and more importantly they did not provide efficient isolation of DNA from the sample. More recently, we have been able to develop electrokinetic techniques that allow nanoscale entities, including high molecular weight (hmw) DNA and nanoparticles, to be isolated from high-conductance (>10 mS/cm) buffer solutions [55-57] and whole blood samples [58]. We were also able demonstrate isolation of virus from blood and fluorescent detection of circulating cell free (ccf) DNA from CLL patient blood samples [59]. Most recent, we were able demonstrate PCR and Sanger DNA sequencing results for ccf-DNA biomarkers isolated by DEP using only 25 µL samples of unprocessed CLL patient blood [60]. The PCR and Sanger sequencing results for the DEP process were equivalent to results obtained using conventional sample preparation of ccf-DNA from 1 mL of CLL patient plasma, and to the “gold standard” DNA sequencing results obtained using an established method for isolating DNA from the leukemic cells of CLL patients that requires 15-20 mL of blood. In the present study, we now compare the DEP isolation of ccf-DNA from CLL patient blood with CLL patient plasma.

2.2 MATERIALS and METHODS

2.2.1 Sample Acquisition

Blood samples were collected from CLL patients and healthy volunteers (IRB#: 080918) in collection tubes containing lithium heparin (BD). For the dielectrophoresis (DEP) experiments, 300 µL of blood was taken from the top of each undisturbed blood sample within 4-5 hours of collection. Plasma was obtained by centrifuging the blood for
10 minutes at 1100 RCF. The supernatant (plasma) was pipetted into a microcentrifuge tube and either frozen or used directly for DEP experiments.

### 2.2.2 Dielectrophoretic (DEP) Isolation of ccf-DNA from Blood and Plasma

New AC dielectrophoretic (DEP) microarray devices (Biological Dynamics, La Jolla, CA) allow the rapid isolation of ccf-DNA and other nanoparticulate biomarkers (ccf-RNA, exosomes, etc.) directly from a small volume of blood and plasma. Figure 2.1 A shows the alternating current electrokinetic (ACE) microarray device (chip), which is approximately 10 mm x 20 mm and contains 1000 microelectrodes that are 60 µm in diameter. The expanded view shows a section of the chip which is fabricated on a silicon base with platinum microelectrodes insulated by SiO₂ and over-coated with a porous hydrogel layer. The expanded view also shows the location of the DEP high-field regions (green) and the DEP low-field regions (red) which form when the AC field is applied. In the first step of the process, a blood or plasma sample containing the ccf-DNA is placed into the microarray device (chip) and an AC electric field is then applied (Figure 2.1 B). At a specific AC frequency and voltage level, the ccf-DNA, which is more polarizable than the surrounding medium, experiences positive DEP (p-DEP) that causes it to concentrate into the DEP high-field regions over the circular microelectrode structures. In the case of blood samples, the blood cells which are less polarizable experience negative DEP (n-DEP) that causes them to move into the DEP low-field regions between the microelectrodes (Figure 2.1 C). Concentration of the ccf-DNA into the DEP high-field regions requires only three minutes, after which a fluid wash removes the blood cells and other blood
components from the microarray (Figure 2.1 D). This is possible because the ccf-DNA in the DEP high-field regions is held more strongly than the blood cells in the DEP low-field regions. Generally, proteins and lower molecular weight biomolecules in the blood are not affected by the AC fields and they are also removed by the washing procedure. After the washing step, the AC field is turned off, at which point the ccf-DNA, if fluorescently stained, can be analyzed on-chip by fluorescence and then the sample can be eluted for

![Figure 2.1 - AC dielectrophoretic (DEP) microarray device and scheme for isolation of ccf-DNA from blood and plasma.](image)

(A) Upper section: the ACE microarray device (chip) used to carry out the isolation of ccf-DNA directly from blood. Expanded view shows the device materials composition: porous gel, platinum microelectrodes, SiO₂ layer and silicon base; and the location of the DEP high-field (green) and the DEP low-field (red) regions when an AC field is applied. Lower section: (B) microarray with whole blood (red circles) containing fluorescent DNA (green dots); (C) application of the AC electric field causing the fluorescent DNA (green dots) to be concentrated in the DEP high-field regions on the microelectrodes, while the blood cells (red circles) move into the DEP low-field regions between the microelectrodes; and (D) the fluidic wash removes the blood cells from the microarray with DNA remaining concentrated in the DEP high-field regions.
The microarray chip itself is contained in a PCB cartridge which forms the sides of the fluidic chamber and is covered with an acrylic window, forming a flow cell with a 25 µL sample volume. A custom-built instrument system (Biological Dynamics) provides the electronic, optical, and fluidic functions under MATLAB software control. Each chip was pre-treated by adding 25 µL of 0.5x PBS (Lonza) to the flow cell and applying a 2 V_{RMS}, 5 Hz sinusoidal waveform for 15 seconds to improve the hydrogel porosity. The 0.5x PBS was then removed and 25 µL of either blood or plasma was added to the flow cell. An 11 Volt peak-to-peak (V_{p-p}), 10 kHz sinusoidal waveform was then applied to the chip for 3 minutes with no fluid flow.

The same electric field was maintained while the chip was washed for 5 minutes at 200 µL/min with 1x TE (Sigma-Aldrich). The electric field was then turned off, allowing captured DNA to diffuse into the 1x TE solution. The 25 µL of fluid was removed within 30 seconds and stored in a microcentrifuge tube. For each CLL patient and healthy donor, this process was repeated 4 times, each time on a new microelectrode device. The 25 µL of eluted sample from each of the 4 runs was combined into a single microcentrifuge tube (100 µL total volume) and stored at 4°C for later analysis.

In order to visualize collection on the microelectrode array, the CLL and healthy donor blood and plasma samples were stained with SYBR Green I fluorescent double-stranded DNA dye (Life Technologies, Carlsbad, CA). One and a half microliters of 100x SYBR Green I was added to 28.5 µL of blood and allowed to incubate at room temperature for 5 minutes. 25 µL of this solution was added to the device and run as described above. After the 3 minutes of electric field collection and 5 minutes of washing, bright field and
fluorescence images of the microelectrode pads were acquired using a CCD camera with a 10x objective, FITC filter, and a 470 nm LED excitation source. DNA with SYBR Green I from these imaged devices was not eluted or used in subsequent analysis.

### 2.2.3 DNA Quantification

After DEP, the isolated ccf-DNA from the blood samples and the plasma samples was eluted and quantified using Quanit-iT PicoGreen (Life Technologies), a double-stranded DNA dye. Each sample was diluted and combined with the PicoGreen reagent, and the resulting fluorescence was measured with a plate reader (Tecan). Standard curve used with PicoGreen assay is included in supplementary material.

### 2.2.4 PCR, Gel Electrophoresis and DNA Sequencing

In order to verify that the collected ccf-DNA was from leukemic B-cells, it was amplified by PCR using Phusion High-Fidelity DNA Polymerase (New England Biolabs). The forward primers used were specific to the VH1, VH3, and VH4 regions, and the reverse primer was specific for the JH region. PCR thermal cycling conditions were a 5-minute initial denaturation at 98 °C followed by 40 cycles of 98 °C denaturation for 15 seconds, 66 °C annealing for 15 seconds, and 72 °C extension for 15 seconds. While CLL blood samples were PCR amplified with each set of VH primers (VH1, VH3, and VH4), the plasma samples were only amplified with the correct VH primer. The PCR product was then analyzed by gel electrophoresis on a 2% agarose gel containing ethidium bromide (Life Technologies). The gels were viewed in a transilluminator and images were captured
using a CCD camera. The images were analyzed with ImageJ software to determine the fluorescence in the region where the main 500-550 bp CLL target fragments should appear, regardless of whether or not a discrete band was observed. Remaining PCR product was cleaned up with the QIAquick PCR purification kit (Qiagen) and sequenced using the Sanger DNA sequencing method [60].

2.2.5 CLL PBMC Genomic DNA Extraction and IgHV Analysis

Peripheral blood mononuclear cells (PBMCs) from CLL patients were isolated by density centrifugation using Ficoll-Hypaque 1077 (Sigma-Aldrich, St. Louis, MO) and suspended in fetal calf serum containing 10% dimethylsulfoxide (Sigma–Aldrich) for storage in liquid nitrogen [63]. DNA was extracted using a Qiagen kit (QIAamp DNA Mini kit, Qiagen, Valencia, CA) according to the manufacturer's instructions and eluted in 30 µL of nuclease-free water. The IgVH gene characterization and mutation status was assessed as previously described [64, 65]. Most PCR products were sequenced directly, although in some cases, amplified products were cloned into pGEM-T (Promega, Madison, WI). Nucleotide sequences were analyzed using the ImMunoGenetic (IMGT) directory (European bioinformatics Institute ImMunoGeneTics Informations System available at http://imgt.cines.fr) (Leukemia 2011 Langerak, Davi, ERIC guidelines) [66]. Sequences with less than 98% homology with the corresponding germline IgVH gene were considered mutated. The heavy chain complementarity-determining region (HCDR3) was determined by the method of Kabat et al [29] as defined by the number of amino acids between codon
94 at the end of framework 3 and the conserved Trp of position 102 at the beginning of framework 4.

2.3 RESULTS and DISCUSSION

2.3.1 DEP Isolation of ccf-DNA

In this study an AC DEP microarray was used to isolate ccf-DNA from eleven chronic lymphocytic leukemia (CLL) patient blood and plasma samples and one normal blood and plasma sample. The study shows that the AC dielectrophoresis technique and microarray allows: (1) ccf-DNA to be isolated directly from 25 µL of unprocessed blood or plasma, (2) on-chip fluorescence analysis of the ccf-DNA in less than five minutes, and (3) elution of the ccf-DNA from the microarray chip for subsequent PCR and DNA sequencing analysis in less than ten minutes. The manipulations for the DEP process comprise two simple steps: the addition of the blood or plasma sample into the microarray device and removal of the eluted sample upon completion of the process. Plasma has the required extra steps of pipetting and centrifugation before the sample is applied to the DEP microarray.

Fluorescence analysis to determine the concentration of ccf-DNA was also carried out after elution from the DEP chip. The results from the DEP process for isolation ccf-DNA from both blood and plasma were then compared to conventional sample preparation process for isolation of genomic DNA from the leukemic B-cells of CLL patients. This process requires a much larger blood sample and several hours to complete before fluorescence analysis, PCR and DNA sequencing of the genomic DNA can be carried out.
Figure 2.2 shows a comparison of the processing time and number of manipulations required for the DEP procedure for blood (A) and for plasma (B), with the CLL Lab procedure for isolating genomic DNA from patient B-lymphocyte cells (C).

Figure 2.2 – Processing times and steps for DEP isolation of ccf-DNA from blood and plasma compared with isolation of genomic DNA from CLL patient B-lymphocytes.

(A) The DEP procedure used to isolate ccf-DNA directly from 25 µL samples of unprocessed CLL patient blood. (B) The DEP procedure used to isolate ccf-DNA directly from 25 µL samples of CLL patient plasma. (C) The procedure used to isolate genomic DNA from CLL patient B-lymphocyte cells starting with 15-20 mL of patient blood.

With regard to the DEP process, the blood sample can be applied to the microarray immediately. However, for this study the DEP isolation was generally carried from 0-3 hours after the blood draw. For the CLL Lab procedure, the processing times include only the actual time necessary to run a specific processing step, i.e. 10 minutes for centrifugation. Additional time is necessary for setting up, carrying out transfers such as
pipetting and for many other manipulations. When this process is performed manually the
additional manipulations can add at least another hour to the total time required for CLL
Lab process, and they also add significantly to the overall cost of the assays.

2.3.2 On-Chip fluorescence detection of ccf-DNA

For on-chip fluorescence detection of the ccf-DNA, SYBR® Green I (Invitrogen) stain is added to the blood samples and plasma samples before the application of the DEP field. After DEP is carried out for three minutes and blood cells (blood samples) and plasma are removed by a fluidic wash, the fluorescently stained ccf-DNA, which is concentrated in the DEP high-field regions (on the microelectrodes), is detected. Figure 2.3 shows the fluorescent image results for ccf-DNA isolated by DEP from a normal blood (Normal-1e B) and plasma sample (Normal-1e P), and five CLL blood (CLL-1e B, CLL-2e B, etc.) and plasma samples (CLL-1e P, CLL-2e P, etc.). On the far right of each fluorescence image is a 3D fluorescence intensity image created by MATLAB, which provides better visualization of the relative amounts of ccf-DNA that were isolated. Overall, the fluorescent DNA levels (3D intensity) were higher in most of the CLL patient samples when compared to the fluorescent DNA levels obtained for the normal blood (Normal-1e B) and plasma (Normal-1e P) sample.
Figure 2.3 – Fluorescence detection of ccf-DNA in five CLL patient blood and plasma samples and one normal blood and plasma sample.
On-chip fluorescence imaging results from 25 µL blood and plasma samples showing SYBR Green stained ccf-DNA that was concentrated into the DEP high-field regions after the DEP field was applied for three minutes. Images of one normal blood (Normal-1e B) and plasma sample (Normal-1e P) and five CLL blood (B) and plasma (P) samples (CLL-1e, CLL-2e, CLL-3e, CLL-4e and CLL-5e). White dotted square areas in the images on the left side are enlarged in the center column images. The right side column shows 3D fluorescence intensity images created by MATLAB, which provide better visualization of the relative amounts of ccf-DNA that were isolated on the DEP high-field areas over the microelectrodes.
2.3.3 Concentration of Eluted ccf-DNA from Blood and Plasma Samples

In additional experiments, Quant-iT PicoGreen (Invitrogen) fluorescence analysis was used to determine the concentration of the ccf-DNA in the CLL and normal blood and plasma samples after elution from the DEP microarray. For these experiments SYBR Green I DNA dye was not added to the blood or plasma samples prior to DEP. Figure 2.4 shows the ccf-DNA concentration results (after DEP) for the eluted blood samples (red bars) and for the eluted plasma samples (yellow bars). No significant correlation could be found between the DEP blood results and plasma results for the CLL samples. This is believed to be due to the elution process for removing the isolated ccf-DNA from the DEP device, which is only semi-quantitative. Since the CLL lab procedure uses genomic DNA isolated from leukemic B cells it does not provide meaningful gauge for ccf-DNA in the blood or plasma. Nevertheless, all of the CLL blood and most of the CLL plasma ccf-DNA concentrations were higher than the normal blood and plasma sample.

2.3.4 PCR and DNA Sequencing Results

The eluted ccf-DNA isolated by DEP from both the CLL blood and plasma samples was amplified using primers for the IgVH1, IgVH3, and IgVH4 regions as described previously [60]. The equivalent of just 5 µL of the original CLL blood or plasma sample (25 µL) was amplified using IgVH specific primers. The correct IgVH PCR amplification products were obtained for all eleven CLL blood and plasma samples, although some bands were weak and secondary bands were also present. It should be pointed out that the CLL lab procedure does not require comparisons to “normal blood or plasma” samples, therefore
the ccf-DNA isolated by DEP from the normal blood and normal plasma samples were not used for PCR or sequencing.

Once the IgVH regions for each of the CLL samples were analyzed using PCR, the resulting PCR products were sequenced and compared to the results previously obtained using an established method performed on genomic DNA obtained from CLL patient leukemic cells. Sanger sequencing was used to verify that the isolated ccf-DNA was...
coming from the leukemia cell population, and that the amplified IgVH regions matched the results obtained by PCR of genomic DNA isolated from B-lymphocytes. Table 2.1 show the DNA sequencing results for all eleven CLL samples; the sequences obtained from the ccf-DNA isolated by DEP from the equivalent of 5 µL of blood and plasma are compared to the “gold standard” CLL patient sequences in the database, which were obtained from isolating genomic DNA from patient B-lymphocyte cells. All eleven CLL patient DNA sequences from the DEP blood samples show perfect homology with the genomic DNA “gold standard” results. Eight of the eleven CLL sequences for the DEP plasma samples show perfect homology with the genomic DNA “gold standard” results. The ccf-DNA from DEP plasma sample CLL-3e shows 98/100% homology, DEP plasma sample CLL-2e showed 84/100% homology and DEP plasma sample CLL-4e did not sequence. Several possible reasons exist for these three CLL plasma sequencing results: (1) less ccf-DNA was present in the plasma samples; (2) less DNA was eluted from the DEP microarray; and (3) PCR is not fully optimized for amplification of very low levels of ccf-DNA.

2.4 DISCUSSION

Ccf-DNA and ccf-RNA have the potential to become important biomarkers for cancer diagnostics and patient management. Ccf-DNA/RNA isolated from blood and/or plasma, constituting a “liquid biopsy,” may serve as an alternative to more invasive tissue biopsies in the detection and analysis of cancer mutations. Unfortunately, the time, complexity, and cost of employing conventional methods to isolate ccf-DNA/RNA from
Table 2.1. DNA sequencing results.
DNA sequencing results for ccf-DNA isolated from CLL blood samples by DEP and ccf-DNA isolated from the same CLL patient plasma samples by DEP process compared with the results obtained using an established method performed on genomic DNA obtained from CLL patient B-leukemic cells.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>DEP Blood</th>
<th>DEP Plasma</th>
<th>Genomic DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V-Gene/Allele</td>
<td>V-Region ID %</td>
<td>V-Gene/Allele</td>
</tr>
<tr>
<td>CLL – 1e</td>
<td>IGHV3-30*03 F</td>
<td>95.14%</td>
<td>IGHV3-30*03 F</td>
</tr>
<tr>
<td>CLL -2e</td>
<td>IGHV1-2*02 F</td>
<td>100.00%</td>
<td>IGHV1-69*01 F</td>
</tr>
<tr>
<td>CLL – 3e</td>
<td>IGHV4-4*07 F</td>
<td>100.00%</td>
<td>IGHV4-4*07 F</td>
</tr>
<tr>
<td>CLL – 4e</td>
<td>IGHV1-8*01 F</td>
<td>100.00%</td>
<td>No Productive gene</td>
</tr>
<tr>
<td>CLL – 5e</td>
<td>IGHV3-21*02 F</td>
<td>88.19%</td>
<td>IGHV3-21*02 F</td>
</tr>
<tr>
<td>CLL – 6e</td>
<td>IGHV3-33*01 F</td>
<td>94.10%</td>
<td>IGHV3-33*01 F</td>
</tr>
<tr>
<td>CLL – 7e</td>
<td>IGHV3-53*04 F</td>
<td>97.19%</td>
<td>IGHV3-53*04 F</td>
</tr>
<tr>
<td>CLL – 8e</td>
<td>IGHV3-64*01 F</td>
<td>94.79%</td>
<td>IGHV3-64*01 F</td>
</tr>
<tr>
<td>CLL – 9e</td>
<td>IGHV4-39*01 F</td>
<td>96.91%</td>
<td>IGHV4-39*01 F</td>
</tr>
<tr>
<td>CLL – 10e</td>
<td>IGHV4-34*01 F</td>
<td>99.30%</td>
<td>IGHV4-34*01 F</td>
</tr>
<tr>
<td>CLL – 11e</td>
<td>IGHV4-39*01 F</td>
<td>100.00%</td>
<td>IGHV4-39*01 F</td>
</tr>
</tbody>
</table>

blood/plasma can limit the use of these procedures, especially for point-of-care (POC) diagnostic applications. This study demonstrates the ability of a DEP microarray device to isolate ccf-DNA directly from a small amount (25 µL) of unprocessed blood or plasma samples. The DEP process comprises only two steps and can be completed in less than 10 minutes. In contrast, most conventional sample preparation processes typically involve obtaining 1-2 mL of plasma from 2-3 mL of blood and subsequently subjecting the plasma
to a series of manipulations to obtain ccf-DNA over the course of 1-2 hours [60]. The present CLL lab process for isolating genomic DNA from B-lymphocytes requires 15-20 mL of blood and is a complex and expensive process that takes several hours to complete. The DEP process enables the rapid use of unprocessed blood or plasma samples which should lead to a significant reduction in the cost and complexity of ccf-DNA isolation relative to conventional methods for isolation of ccf-DNA.

The DEP procedure for blood samples provides PCR and DNA sequencing results comparable to conventional sample preparation results [60], and to results obtained using an established method performed on genomic DNA. An additional advantage of using the DEP process is the ability to carry out fluorescence detection of ccf-DNA within minutes of application of the blood/plasma sample to the chip. In this study, CLL samples possessed higher fluorescence intensity levels for SYBR® Green stained ccf-DNA concentrated in DEP high-field regions than the normal blood samples. In many cases the fluorescence intensity levels of the CLL samples were substantially higher than those of the normal sample. Ultimately, the use of “on-chip” fluorescence to rapidly determine ccf-DNA levels in clinical blood samples could provide a first-level “alarm” for POC diagnostics. This could provide an indication of the presence or occurrence of an abnormality that could require further monitoring. In the case of solid tumors, researchers have demonstrated a correlation between ccf-DNA levels in patient plasma and survivability for lung and colon cancers [61, 62]. However, the isolation of ccf-DNA in these studies required long and involved processes.
In summary, the DEP technique shows considerable potential for enabling rapid, simple and cost-effective “liquid biopsy” and POC cancer diagnostics. In addition, the DEP technique may become a powerful tool for biomedical research. Currently, the true in vivo nature and actual levels of ccf-DNA/RNA, exosomes and other nanoparticulate biomarkers in blood are not well known. The ability to rapidly isolate, in their unperturbed states, the cellular nanoparticulates released into the bloodstream by injured, necrotic and transformed cells is critical to a better understanding of the disease process itself. Unquestionably, conventional sample preparation procedures, which involve processing plasma from blood and subsequently subjecting plasma to numerous time-consuming/labor-intensive physical manipulations, may lead to loss and degradation of the biomarkers. The use of this DEP technique for rapid isolation of ccf-DNA/RNA directly from blood samples promises to provide biomarkers in their unperturbed state and this may, in turn, enable researchers to deliver better diagnostic tools and research applications.

2.5 ACKNOWLEDGMENTS

Chapter 2, in part, is a reprint of the following manuscript: Dielectrophoretic Isolation and Detection of Cancer Related Circulating Cell Free DNA Biomarkers from Blood and Plasma. Co-authors Avery Sonnenberg, Jennifer Marciniak, Elaine Skowronski, Sareh Manouchehri, Laura Rassenti, Emanuela M. Ghia, George F. Widhopf II, Thomas J. Kipps, Michael J. Heller. This has been published in Journal of Electrophoresi
2.6 REFERENCES


CHAPTER 3
Dielectrophoretic Recovery of DNA from Plasma for the Identification of Chronic Lymphocytic Leukemia Point Mutations

3.1 INTRODUCTION

B-cell chronic lymphocytic leukemia (CLL) is one of the most common forms of leukemia in human adult [1, 2]. In the United States alone more than 14,000 new cases are diagnosed annually, with over 4,500 deaths every year [1, 2]. CLL is rarely curable and its clinical course is heterogeneous [1, 3]. Some patients are free of symptoms for many years, during which time treatment is typically not necessary. For others the disease is relatively aggressive and requires therapy soon after diagnosis. Because standard therapies are associated with potential morbidity and are not considered curative, current recommendations are to withhold treatment until the patient manifests disease-related complications or clear evidence of disease progression [2, 4, 5].

One of the main factors that influences the clinical course of CLL is the acquisition of mutations in genes that may expedite disease progression. Therefore, the early identification of these mutations is essential for CLL diagnostics in the clinical setting [3, 4, 6, 7]. Mutations in TP53, NOTCH1, and SF3B1 genes can occur in ~5-20% of CLL patients at the time of diagnosis. [4, 8, 9] These genes have been shown individually to have significant correlations with poor prognosis and treatment resistance in several studies.[8, 9, 10] Although these mutations may be absent at initial diagnosis, there is a high probability of developing new high-risk genetic lesions during the course of the disease (>25% at 10-years) [11], and occurrence of the high-risk genetic lesions over time can directly affect survival time [11, 12]. These genetic lesions give us clues as to which
divergent clinical prognosis is possible for a given CLL patient and can be used to guide the management of CLL [9, 11, 12]. Detection of these genetic lesions can be performed by extracting DNA from B-CLL cells, followed by targeted PCR, gel electrophoresis, and DNA sequencing. However, mutations can also be detected in circulating cell-free (ccf) DNA isolated from CLL plasma or CLL blood [13, 19, 20].

Ccf-DNA is now becoming an important biomarker for early detection of cancers and residual disease and can be used for therapy monitoring and cancer management [1, 13-18]. The use of ccf-DNA isolated from plasma (liquid biopsy) for the detection of cancer mutations may become a better alternative to the more invasive tissue biopsies [1, 14, 15]. Some plasma proteins inhibit PCR amplification and require that the DNA be recovered from plasma in order to perform PCR analysis to determine the sequence. Unfortunately, recovering the DNA from human plasma samples is a challenge that prohibits the use of ccf-DNA for point-of-care (POC) diagnostics. The conventional techniques for separation of ccf-DNA from plasma are highly complex and time consuming; and usually require at least 1 mL of plasma. Traditional methods used to recover DNA from plasma involve several extraction steps including the introduction of special solvents [23, 24]. This involves a large number of manipulations including multiple steps of pipetting and filtration that can shear DNA into smaller pieces. These manipulations increase the chance of human error, and can also cause degradation in the quantity and quality of the ccf-DNA [16, 23, 24].

To reduce DNA degradation and simplify the recovery process we present a new electrokinetic method that uses dielectrophoresis (DEP) to recover ccf-DNA from
undiluted human plasma. Recently an enabling technological advance in the design of the DEP microelectrode chips allows DEP to be performed in high conductance media such as whole blood, plasma, and serum. [21-24]. This allows for the rapid isolation of nanoscale entities including high molecular weight (hmw) DNA within 20 minutes.

Dielectrophoresis uses an alternating electric field to create a separation force based on differences between the dielectric properties of the ccf-DNA and the surrounding plasma fluid [25]. When dielectric particles such as DNA are placed in an electrical field, they become polarized and create a dipole moment. This dipole moment is frequency dependent and its magnitude depends on the polarizability of the DNA relative to the plasma which can be described by the Clausius-Mossotti factor. In a non-uniform alternating current (AC) electric field polarized particles experience this DEP force. A particle with a higher polarizability than its suspending medium will experience a positive DEP force which pushes it towards the region with higher electric field strength (high field) and the particle with lower polarizability compared to its medium, will be driven to the area with lower electric field strength (low field) by the force of negative DEP [26]. Using an appropriately designed microelectrode device, dielectric particles can be concentrated in the areas of high or low electric field. In this study we used microelectrode arrays produced by Biological Dynamics (San Diego, CA) to trap and isolate ccf-DNA from CLL plasma samples using DEP. Sufficient amounts of ccf-DNA can be recovered using this method to be used to identify the characteristic point mutations in genes associated with CLL.
3.2 MATERIALS and METHOD

3.2.1 Sample Acquisition

Peripheral blood mononuclear cells (PBMCs) and plasma samples were collected from 12 patients who satisfied diagnostic and immunophenotypic criteria for common B-cell CLL [5]. These individuals have signed the written informed consent in compliance with the Declaration of Helsinki. PBMCs were isolated by density centrifugation using Ficoll-Hypaque 1077 (Sigma-Aldrich, St. Louis, MO) and suspended in fetal-calf serum containing 10% dimethylsulfoxide (Sigma–Aldrich) for storage in liquid nitrogen [27]. Samples each contained >90% CLL cells.

DNA was extracted from the viable frozen CLL PBMCs (QIAquick PCR purification kit, 70 Qiagen). We amplified and sequenced exons 14 and 15 of SF3B1 gene, exons 5 through 8 of TP53 gene and exon 34 of NOTCH1 gene. Parameters for PCR amplifications were as previously described [28]. The products were purified with QIAamp DNA minikit (Qiagen) and were confirmed by 2% agarose gel. Sanger sequences were compared to the corresponding germline RefSeq using the software Mutation Surveyor® v.4.0.6 (SoftGenetics, State College, PA).

Frozen plasma samples collected from the same 12 patients were stored in -80 °C and used to extract ccf-DNAs.

3.2.2 DNA Extraction using Dielectrophoresis

AC electrokinetic microarray chips, from Biological Dynamics, were used for isolation of ccf-DNA from the plasma using the Biological Dynamics Generation 4 Elution
These microarray devices contain 1000 platinum microelectrodes which are fabricated on a silicon base and coated with a hydrogel (PolyHEMA) layer. Each microarray chip is contained within an acrylic microfluidic cartridge that allows the chip to hold approximately 25 µL of fluid. 49 µL from each plasma sample was stained with 1 µL of 100x diluted YOYO®-1 Iodide (Molecular Probes® -ThermoFisher Scientific) and incubated for 2 minutes. 25 µL from each stained sample was then placed into the microarray device. The AC electric field was applied to the microarray chip with a magnitude of 12 volts peak-to-peak (V_{p-p}) and a sinusoidal waveform of 15-KHz for about 10 minutes.

At these DEP parameters, the ccf-DNA is more polarized than the plasma and so experiences a positive DEP force which causes it to concentrate around the edge of the circular microelectrodes in the DEP high field region. A top view of the microarray chip for each sample showing an image of fluorescent DNA collected on the electrode edges is shown in figure 1. Maintaining the chip at the same voltage and frequency, each chip then was washed at 20 µL/min with 1x TE buffer (Sigma-Aldrich) for an additional 10 min. The buffer wash and the electric field were then turned off. The DNA collected at the array electrodes was allowed to diffuse up into the TE buffer by Brownian motion for approximately 1 min. The wash buffer containing the collected ccf-DNA (~20 µL) was then eluted into a micro-centrifuge tube and stored at 4 °C for further analysis.

3.2.3 Quantification of Collected DNA Fluorescence Intensity

A custom MATLAB script was used to quantify the fluorescence intensity of the isolated DNA on the edge of the electrodes. 3D graphs with the X and Y axes representing
the physical dimensions of the image and the Z axis showing the fluorescence intensity were created for each sample (Figure 3.1).

### 3.2.4 DNA Quantification of DEP Recovered DNA

The ccf-DNA recovered by DEP from the plasma samples were separated into three groups according to the presence of specific mutations previously identified on DNA samples extracted from CLL cells: *SF3B1* mutated (samples 1 through 5), *NOTCH1* mutated (samples 6 - 9) and *TP53* mutated (samples 10-12). For DNA quantification, 2 µL of each eluted sample was combined with the Qubit® dsDNA HS Assay Kit (Invitrogen™) and was quantified with Qubit Fluorometric Quantitation (ThermoFisher Scientific).

### 3.2.5 PCR, Gel Electrophoresis, and Sanger Sequencing

In order to verify the presence of the specific mutations in each sample, 5 µL of the eluted DNA, equivalent to approximately 5 µL of plasma sample, was amplified by PCR using Phusion High–Fidelity DNA polymerase (New England Biolabs) and 5 sets of forward and reverse primers to specifically amplify the above mentioned exons in each gene (Table 3.1). The same PCR cycling conditions were used for all samples and are as follows. Initial denaturation was performed at 98 °C for 5 min followed by 40 cycles of denaturation (98 °C, 15 sec), annealing (67 °C, 15 sec), and extension (72 °C, 15 sec). The PCR products then were analyzed using gel electrophoresis on a 2% agarose gel (E-Gel® EX, Invitrogen™). The gels were analyzed under a UV transilluminator to determine if the
amplicons showed the correct band size. The samples which showed a distinct band were then identified and sequenced using Sanger sequencing (Eton Bioscience Inc.). Purification of the PCR product was performed at the sequencing location.

3.3 RESULTS and DISCUSSION

In this study 12 archived plasma samples from CLL patients were analyzed. We categorized these samples into three different groups based on the presence of specific gene mutations. Ccf-DNA from the plasma samples was successfully collected on the DEP microarray as shown in figure 3.1. As seen in both the fluorescent image and its associated fluorescence intensity graph, samples 1, 2, 6, 8, 11, and 12 show larger amounts of ccf-DNA concentrated on the edge of the electrodes in the high DEP field region. For the other samples it was concluded that there was insufficient DNA in the original plasma to be detected.

The concentration of DNA eluted from the DEP microarray chips was quantified using the Qubit Fluorometric Quantitation system and sis shown in Figure 3.2. The concentration of DNA varies from sample to sample which is expected as there is natural variability amongst individual patients and samples were collected from patients at different stages of the disease.
Figure 3.1 - Fluorescence detection of ccf-DNA collected by DEP in twelve archived CLL plasma samples.

5000X diluted YOYO 1 fluorescent dye was added to each sample to enable us to obtain these mono-color fluorescence images. These images were taken after 15 minutes of applying DEP and after the washing step removed the bulk plasma. The ccf-DNA stained by fluorescent dye concentrated in the microarray DEP high field region as seen in samples 1, 2, 6, 11, and 12. For each fluorescent image a 3-dimensional fluorescence intensity plot has been created in order to quantify the fluorescence intensity of the isolated ccf-DNA.
At this early stage of DEP chip development there can be variability in chip electrode performance which could also have contributed to the observed variation in the amount of captured DNA collected on the electrodes. As seen in figure 3.2, no usable DNA was recovered from sample 3 due to a technical malfunction. However, DNA at a concentration ranging from 37.3-472 ng/mL was recovered for each of the other samples. Five µL of each eluted sample was used for PCR amplification using the specific primer sets shown in table 3.1.

**Figure 3.2– Quantification of DNA recovered from the DEP microarray chips.**
2 µL of each sample eluted off the chip was used to quantify the DNA. The amount of DNA recovered varied from sample to sample ranging from 37.3 ng/mL to 472 ng/mL. No DNA was recovered from the chip containing sample CLL 3 due to a malfunction.
Table 3.1 – List of the PCR primer sets used for each sample.

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Gene</th>
<th>Forward Primer 5’-&gt;3’</th>
<th>Reverse Primer 5’-&gt;3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>P53 exon5-6</td>
<td>GACTTTCAACTCTGTCTCCTT</td>
<td>CCAGAGACCCCAGTTGCAA</td>
</tr>
<tr>
<td>10, 12</td>
<td>P53 exon7-8</td>
<td>AAGGCACCTGGCCTCAT</td>
<td>AAGTGAATCTGAGGCATAAC</td>
</tr>
<tr>
<td>6,7,8,9</td>
<td>Notch1 exon34</td>
<td>GTGACCGCAGCCCAAGTT</td>
<td>AATGCGGGCGATCTGGGACT</td>
</tr>
<tr>
<td>2,5</td>
<td>SF3B1 exon14</td>
<td>TCTGTTTATGGAATTATGGAA</td>
<td>GGGCAACATAGTAAGACCTGT</td>
</tr>
<tr>
<td>1,4</td>
<td>SF3B1 exon15</td>
<td>TTGGGGCATAGTTAAAACCTG</td>
<td>AAATCAAAAGTAAATTGTGGA</td>
</tr>
</tbody>
</table>

Gel electrophoresis analysis showed the correct sized band of PCR amplification products for 7 of the CLL plasma samples (CLL- 1, 2, 6, 8, 10, 11, 12). The intensity of these different bands was seen to have variability between different samples. Samples with a higher levels of ccf-DNA collected from the DEP chip had a higher chance of successfully amplifying. CLL samples 1, 2, 6, and 11 had ≥100 ng/mL of ccf-DNA collected from the DEP chip and all successfully amplified. Only CLL samples 8, 10, and 12 which had ≤100 ng/mL of ccf-DNA amplified. This trend can also be seen in the fluorescent images of each chip where chips showing higher levels of fluorescence also had a better chance of successfully amplifying with the exception of CLL-8 and CLL-10. Several possible reasons for some samples not amplifying include less ccf-DNA present in the original plasma samples; less DNA eluted from the microarray chips, and the PCR protocol not being fully optimized for amplification of low concentrations of ccf-DNA. Future optimization of the design and manufacturing of the microarray devices may lead to higher amounts of DNA recovered from the chips.
After gel electrophoresis the remainder of the successfully PCR amplified DNA were sequenced using Sanger sequencing. The results were compared with the sequencing results obtained from PCR amplification of DNA extracted from CLL cells. Five samples (CLL- 1, 2, 8, 10, and 11) exhibited matching sequences. These results reveal that at least some of the ccf-DNA isolated by DEP was from the leukemia cell population. Figure 3.3 shows the sequencing results for representative mutations in each gene. The sequencing results from the DNA and the ccf-DNA revealed a heterozygous missense mutation (E622D) in SF3B1 gene in the CLL-2 sample, a heterozygous deletion in NOTCH1 gene in the CLL-8 sample, and a homozygous missense mutation (520A>T) in TP53 gene in the CLL-11 sample.

3.4 CONCLUSION

Frozen CLL plasma samples from 12 different patients were obtained from the UCSD Moores Cancer Center bio-repository for this study. Specific mutations in these samples were identified after collecting CLL cells from patients, extracting DNA, and then amplifying and sequencing specific exons in the SF3B1, NOTCH1 and TP53 genes. In this study we verified the existence of specific mutations in such genes using a novel microarray chip capable of performing dielectrophoresis in highly-conducive media. This electrokinetic technique rapidly isolated ccf-DNA from plasma samples. DEP was successfully applied to all CLL samples using only 25 µL of plasma. After a microfluidic washing step using 1x TE buffer to remove the bulk plasma, the concentrated ccf-DNA was recovered from each microarray chip. DNA was recovered in a sufficient amount to allow for PCR amplification and sequencing for 5 of the 12 CLL patient samples.
Figure 3.3 – Sequencing results for three archived CLL samples.

The sequencing results obtained by sequencing DNA isolated from CLL cells are shown in the top panel and the sequencing results obtained from ccf-DNA isolated by the DEP technique are shown in the bottom panel. The sequencing results from the DNA and the ccf-DNA in the CLL-2 sample shows a heterozygous missense mutation (E622D) in the SF3B1 gene, in the CLL-8 sample a heterozygous deletion in the NOTCH1 gene, and in the CLL-11 sample a homozygous missense mutation (c.520A>T) in the TP53 gene.
The sequencing results matched the ones obtained from DNA extracted from CLL cells. The CLL-1 and CLL-2 samples showed mutations in exon 15 and 14 of the SF3B1 gene, respectively, the CLL-8 sample showed a 2 bp deletion in exon 34 of the NOTCH1 gene, and the CLL-10 and CLL-11 samples showed missense mutations in exon 7 and 5 of the TP53 gene, respectively. The PCR amplification was not successful for most samples where less than 100 ng/mL of ccf-DNA was recovered from the DEP chip. These samples may have come from patients who had an insufficient amount of ccf-DNA in circulation to reach this threshold for consistent detection. In general, the amount of DNA that can be recovered from plasma is much less than what can be recovered directly from isolated CLL cells. There is also variability in the performance of the chips at this early stage of development that could contribute to the observed overall variability.

To the best of our knowledge, this work is the first study using dielectrophoresis to collect DNA from plasma to detect specific cancer related mutations. Future work will continue to optimize this DEP technique and to make it available as a simple and rapid means to perform liquid biopsies for POC cancer diagnostics and treatment monitoring.

3.5 ACKNOWLEDGMENTS

Chapter 3, in part, is in preparation for submission for publication as:
Dielectrophoretic Recovery of DNA from Plasma for the Identification of Chronic Lymphocytic Leukemia Point Mutations. Co-authors. Sareh Manouchehri Stuart Ibsen, Jennifer Wright, Laura Rassenti, Emanuela M. Ghia, George F. Widhopf II, Thomas J. Kipps, Michael J. Heller.
3.6 REFERENCES


