Isolation and Detection of Cancer Related DNA Nanoparticulate Biomarkers Directly from CLL Patient Blood “Rapid Sample to Sequence Diagnostics”

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ABSTRACT

We have now demonstrated a dielectrophoretic (DEP) based approach that allows a full range of cfc-DNA biomarkers from chronic lymphocytic leukemia (CLL) patients to be isolated directly from a small volume of unprocessed whole blood. Using a DEP microarray device, cfc-DNA from 50 uls of patient blood is separated and concentrated into DEP high-field regions in about 10-15 minutes. Blood cells, serum proteins and low molecular biomolecules are quickly removed by a fluidic wash. The cfc-DNA concentrated on the DEP microelectrodes is first analyzed by fluorescence, and then eluted with a fluidic wash for subsequent PCR and sequencing analysis. In a majority of CLL samples, fluorescent stained cfc-DNA was clearly observed on the DEP microelectrodes, while little or no fluorescence was observed for the normal blood control samples. Eluted cfc-DNA (equivalent to 2ul-5ul of original blood sample) was then PCR amplified using VHL specific primers, which verified the CLL patient type for 29 of 35 CLL samples. The CLL patients amplified DNA was then sequenced and correlated with previous patient specific sequencing results from DNA originally isolated from white blood cells.

Keywords: dielectrophoresis, DNA biomarkers, cancer, blood

1 INTRODUCTION

While the potential medical applications of nanotechnology are rapidly growing, a number of issues still need to be resolved before nanomedicine moves from the lab to the bedside. One very important challenge in nanomedicine will be the detection of early disease nanoparticulate biomarkers, in particular cell free circulating (cfc) DNA and RNA. The ability to rapidly detect low levels of cfc-DNA/RNA and other nanoparticulate biomarkers directly in blood would represent a major advancement for early cancer detection and screening, chemotherapy monitoring and residual disease detection. Cfc-DNA and cfc-RNA are potentially important biomarkers for early cancer detection [1]. These biomarkers are generally high molecular weight (hmw) DNA/RNA nanoparticulates that are released into the blood stream by tumor cell necrosis [2]. Unfortunately, it remains a challenge to isolate and detect cfc-DNA and other early disease biomarkers directly in complex samples like blood [3]. Often, the detection of early disease biomarkers is a statistical problem requiring that a relatively large sample (1-5ml) be processed. Even though highly sensitive detection technologies (PCR, sequencing, etc.) are available for subsequent analysis [4], the sample preparation process adds considerable time, labor and expense to the diagnostic assay. Furthermore, sample preparation (blood to plasma centrifugations, filtration, etc.) can also cause considerable degradation and loss of hmw-DNA.

Thus, there is a critical need for novel robust technology, which will allow a variety of important nanoscale biomarker entities to be manipulated, isolated and rapidly detected directly from whole blood and other biological samples. DEP is a separation technique which uses AC electric fields to manipulate cells and nanoparticles. While high resolution separation of cells, bacteria, virus, and DNA has been carried out by DEP, serious performance limitations have prevented the technology from being used for practical applications. In particular, DEP’s limitation to low ionic strength (low conductance) solutions requires that blood be processed and diluted 10-100 fold before separation [5]. More recently, using microarray devices we were able to demonstrate high conductance (HC) DEP that allowed both hmw-DNA nanoparticulates and nanoparticles to be manipulated, isolated and detected under high ionic strength conditions [6-8]. We have also been able to demonstrate, using HC-DEP microarrays, the rapid isolation and detection of hmw-DNA and nanoparticles in undiluted whole blood [9], and the isolation and detection of cancer related cfc-DNA directly from small amounts of chronic lymphocytic leukemia (CLL) patient whole blood.
We now report some preliminary results on the isolation, fluorescent detection and PCR and sequencing analysis of cfc-DNA isolated by DEP from 50ul samples of CLL patient whole blood. HC-DEP sets the stage for new “seamless” sample to answer diagnostic systems which will allow a variety of important nanoscopic biomarkers and drug delivery nanoparticles to be rapidly isolated and analyzed from clinically relevant amounts of complex un-diluted clinical and biological samples.

2 RESULTS AND DISCUSSION

Figure 1 shows the basic scheme for the DEP separation of cfc-DNA in whole blood using a microarray device. Figure 1A shows the DEP microarray device with whole blood (red and white cells) containing cfc-DNA (green dots). Figure 1B shows the DEP separation of cfc-DNA into the high-field regions (represented as domes) where it is held firmly on the microelectrodes, and blood cells moving into the low-field regions between the microelectrodes where they are held less firmly. A fluidic wash now removes the blood cells while the cfc-DNA remains in the high field regions (Figure 1C). The cfc-DNA can then be detected by fluorescence (on-chip) and/or eluted and further analyzed by PCR and sequencing. Figure 2 shows the DEP microarray device which was used to carry out the isolation and detection of cancer related cfc-DNA from CLL patient whole blood samples. The DEP microarray device was designed and fabricated by Biological Dynamics (La Jolla, CA). Some experimental results showing the isolation and fluorescent detection of cfc-DNA from CLL cancer patient blood is show in Figure 3. Using the DEP microarray device, cfc-DNA from 50 uls of the CLL patient whole blood was separated and concentrated into DEP high field regions in about 10-15 minutes. At DEP frequencies of 5-10 kHz the SYBR green fluorescent-stained cfc-DNA separates from the blood and becomes highly concentrated at the DEP high field regions over the microelectrodes (as seen in Figure 3 A & B). In this preliminary study we then demonstrated using the Biological Dynamics DEP microarray devices, that after the cfc-DNA from 50 uls of patient blood was separated and concentrated into DEP high field regions, and fluorescence analysis was carried out, the concentrated cfc-DNA could be eluted from the DEP microarray by a fluidic wash in 1-2 minutes for PCR and sequencing analysis.
Figure 2 – DEP microarray device (chip) designed and fabricated by Biological Dynamics (La Jolla, CA). (A) Shows the actual 400 site microarray which is 10mm x 25mm in size. (B) Shows a section of the microarray with twelve platinum microelectrodes which are 80um diameter. (C) Shows DEP high field (positive DEP) and low field (negative DEP) areas which are produced on the microarray when the AC field is applied. (C) Shows a cross section of one of the platinum microelectrodes which is over-coated with a hydrogel layer [6, 7, and 8].

Figure 3 – Shows a section of the DEP microarray device with fluorescent stained cfc-DNA isolated from CLL-1 patient (A) and CLL-2 patient (B).

Figure 4 Shows the PCR amplification results (514 bp amplicons) from the DEP isolated cfc-DNA for three CLL patients (patient X, patient Y and patient Z) which correctly identified the VH family gene (VHL 1, 2, 3, 4, 5, 6). The PAGE gel shows: 1. DNA Ladder; 2. Patient X – VH1 primers; 3. Patient X – VH3 primers; 4. Patient X – VH4 primers; 5. Patient Y – VH1 primers; 6. Patient Y – VH3 primers; 7. Patient Y – VH4 primers; 8. Patient Z – VH1 primers; 9. Patient Z – VH3 primers; and 10. Patient Z – VH4 primers. The three patients were correctly identified as VHL type 1.

In a majority of CLL samples, fluorescent stained cfc-DNA was clearly observed on the DEP microelectrodes, while little or no fluorescence was observed for the normal blood control samples. Eluted cfc-DNA (equivalent to 2ul-5ul of original blood sample) was then amplified using VHL specific primers, which verified the CLL patient type for 29 of 35 CLL samples (see Figure 4). The CLL patients amplified DNA was then sequenced and correlated with previous patient specific sequencing results from DNA originally isolated from white blood cells (Dr. Thomas J. Kipps Lab). The cfc-DNA from CLL patients appears to be high molecular weight in that VHL 514 bp amplicons could be readily produced by PCR. Overall, the use of DEP devices for rapid isolation of cfc-DNA directly form a small volume of blood may have considerable potential as a
noninvasive point of care (POC) approach for the
detection of incipient, residual, and recurrent cancer.

3 CONCLUSIONS

The analysis of cell free circulating (cfc) DNA continues to become more widely used for cancer diagnostics and management. Cfc-DNA can range from nucleosomes and high molecular weight DNA nanoparticles to 200bp fragments. Generally, cfc-DNA for “liquid biopsies” is isolated form plasma using relatively long and involved processes. The ability to now rapidly isolate and detect cancer and other disease related cfc-DNA, cellular nanoparticles (RNA exosomes, mitochondria, etc.) directly in blood, plasma, serum and biological buffers will be important for many future clinical diagnostic applications. This study demonstrates the potential clinical relevance of new DEP technology by showing the rapid isolation and detection of SYBR Green stained cfc-DNA from whole blood samples from Chronic Lymphocytic Leukemia (CLL) patients, and subsequent PCR and DNA sequencing analysis. Finally, the results from the DEP microarray devices demonstrating the rapid detection of low levels of cfc-DNA in whole blood patient samples shows considerable promise for cancer patient management and diagnostics. Overall the results of this study support the enormous potential of DEP as a “seamless sample-to-answer” technique for the rapid detection of cfc-DNA and other nanoparticulate biomarkers directly from blood and other complex biological samples.

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