STAGING THE CLINICAL STATUS FROM BLOOD OF CANCER PATIENTS BY CHIP-BASED CELL ENUMERATION FOLLOWING TARGETTED REMOVAL OF NORMAL CELLS

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ABSTRACT

Even though an agreed phenotypic definition of circulating tumor cells (CTCs) remains elusive in the literature, many current detection technologies isolate candidate cells based on molecular recognition of cellular epitopes that may not accurately predict CTC load. Rather than using such an epitope specific "positive-capture" strategy, we present a chip-based, centrifugal microfluidic platform integrating "negative-capture" magnetophoretic removal of normal white blood cells (WBCs) from a sample and subsequent, array-based enumeration of individualized, (untagged) abnormal cells. We compared the numerical recovery of cells on the array with the status of the donor patient, showing that the chip can has the potential to indicate the oncogenic severity of the blood donor.

KEYWORDS: Lab-on-a-Disc, Magnetic Selection, Magnetophoresis, CTC detection

INTRODUCTION

With advances in modern therapies, the prognosis for patients presenting with cancer can be positive in cases where malignancies are detected early. The detection and / or enumeration of CTCs in patient blood is one of the most promising, minimally invasive diagnostic methods to have emerged from translational medicine in recent years [1]. Isolation of CTCs from a sample is generally mediated through antibody binding to a target outer-membrane epitope and subsequent removal of bound cells from the sample or immobilization of cells to a solid phase; alternatively, separation of CTC candidates by size exclusion has been implemented. However, such methods are limited by the inherent heterogeneity within CTC populations. For example, the most common epitope targeted is EpCAM, an exclusively epithelial marker. Detection of EpCAM expressing cells in blood is often used as part of a positive signature for a CTC. Yet, some CTCs do not express EpCAM or other common markers such as cytokeratin. Indeed, EpCAM is down-regulated during epithelial-to-mesenchymal transition (EMT); also in many carcinomas EpCAM is under-expressed or not expressed at all [2].

In the current work, we present a strategy to overcome the inherent heterogeneity of CTCs by using a negative isolation mode rather than the epitope specific positive isolation described above. In this mode, instead of targeting CTCs directly, normal WBCs are bound to paramagnetic beads and isolated to a magnetic locus upstream of a cellular detection array in a centrifugal chip. Hence, as the cells naturally found in blood are excluded, only abnormal cells that escape binding will resolve to the detection array. The higher the number of cellular events recorded at the detection array will then provide an indication as to the extent of CTC load within a blood sample. There is a minor loss of candidate cells which become entrained in the collective migration of abundant tagged cells towards the lateral magnets; however, a sufficient number of candidate cells resolves to the detection array to provide a diagnostic indication of load.

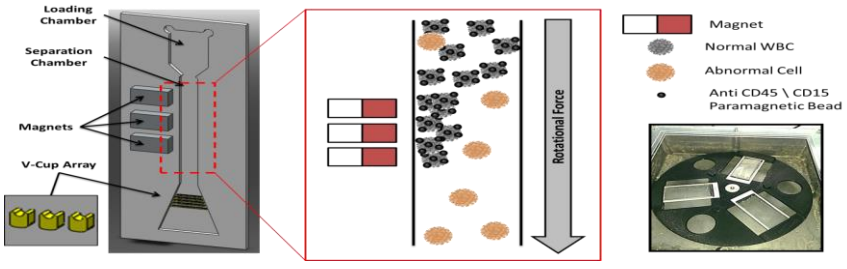

Figure 1: Schematic of the negative isolation blood analysis chip featuring the chambers for loading, magnetic separation and detection in a V-cup array. The separation chamber (red hatched box) is shown in more detail, and demonstrates the sequestering of cells expressing CD15 and CD45 from the sample. Un-deflected cells migrate into the V-cups under the influence of the radial force.

THEORY

Our group has previously shown the use of centrifugo-magnetophoresis as an efficient method for isolating cells of interest from a blood sample [3, 4]. In the here presented system, centrifugo-magnetophoresis is used to immobilize healthy WBCs within the fluidic chamber of the centrifugal chip after incubation of processed blood sample with paramagnetic beads targeting both CD15 and CD45. These markers are absent in abnormal cells which are thus driven by the radial, centrifugal force to the outer region of the chip where an array of scale-matched traps aligns them in a regular grid featuring single-occupancy distribution (Fig. 1). The array consists of 7410 individual posts, each shaped such that only a single cell can occupy the cup-like indentation at the top (Fig. 1). As the specificity is based on removal of phenotypically well-defined healthy WBCs, our system circumvents the issue of CTC heterogeneity, regardless of epitope expression or cell size. As negative selection is used, no further characterization of the cells occupying the cups is required (i.e. fluorescent measurements were not used) to identify the cells as candidate CTCs. Hence, the occupancy of the array is measured simply by optically scoring each cup as occupied or non-occupied.

EXPERIMENTAL

Whole blood was isolated from either healthy volunteers or patients harboring primary (ovarian and melanoma) or recurrent (ovarian) tumors. Where indicated, whole blood was spiked with LnCAP cells pre-stained with Hoechst 33342 to simulate abnormal cells. RBCs were removed using hypotonic lysis, and the remaining nucleated cells isolated by centrifugation, re-suspended in 500 µl of resuspension buffer, and enumerated. paramagnetic beads functionalized against CD15 and CD45 (Life Technologies) were added to the samples and rotated for 20 minutes at 4°C. Chips were then loaded with a volume of sample representing the nucleated cell load equivalent to 2.5 µl of original whole blood, and rotated at 10 Hz for 45 minutes. The V-cup array was visualized using optical brightfield (or fluorescent where indicated) microscopy to quantitate the occupancy of the array.

RESULTS AND DISCUSSION

To demonstrate the operation and efficiency of the negative-isolation and cellular distribution chip, Fig. 2 shows the results of an experiment where 50 cultured LnCAP cells stained with Hoechst 33342 were spiked to a healthy blood sample and processed through the chip as described. In this experiment, a total of 33 cells were retrieved on the V-cup array; composed of all 30 LnCAP cells (as identified by their specific stain) and a remainder of only 3 being (normal) WBCs, despite their huge abundance in the original sample. These data demonstrate a 90% specificity and a 60% sensitivity for the detection of abnormal cells.

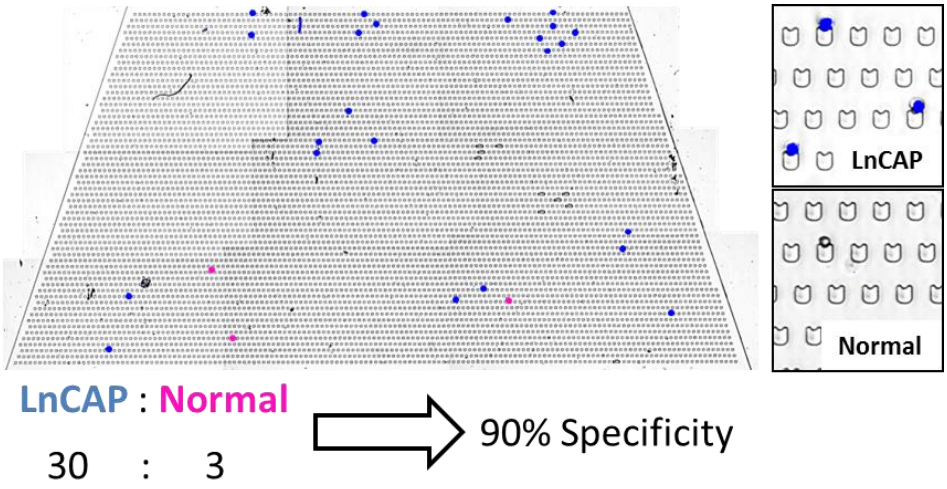

Figure 2: Identification of LnCAP cancer cells spiked to a healthy blood sample using the blood analysis chip. The full V-cup array is shown with locations of LnCAP cells (blue) and normal WBCs (red) marked for clarity. Inserts are zoomed images of LnCAP and normal cells in V-cups.

Blood samples from melanoma and ovarian cancer patients with either primary or recurrent tumors were processed identically (without cellular spikes) and compared to a healthy blood sample by brightfield observation of the occupancy of the V-cup array (Fig. 3). As expected, blood from the healthy volunteer resolved no cellular events to the

V-cups following processing while the all samples from cancer patients exhibited events on the array (Fig. 3i). Furthermore, the number of events was markedly higher (Fig. 3ii) in the sample from the patient with recurrent tumors (170 cells μl^{-1}) than in the sample from a patient a primary tumor (43 cells μl^{-1}).

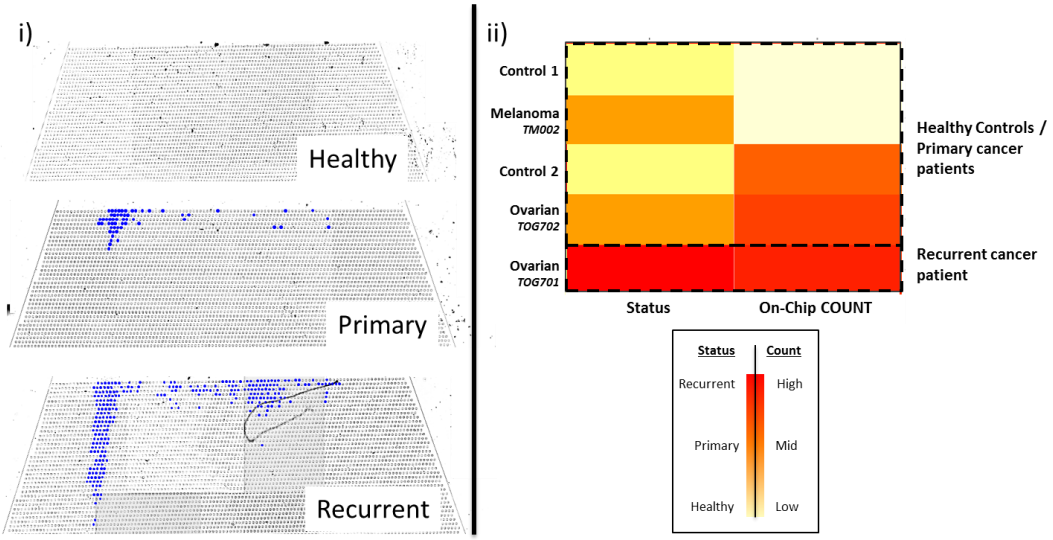

Figure 3: (i) Abnormal cell identification in patient-sourced blood samples. Samples from healthy patients did not display any detectable cells in V-cups, but patients with primary cancer and recurrent tumor harbored cells that populated the capture array. Furthermore, patients with recurrent tumor showed a higher level of capture. (ii) Analysis of cell count read on the V-cup array relative to the status of the blood donor.

CONCLUSION

We introduced a stopped-flow, centrifugo-magnetophoretic strategy to identify and enumerate CTC candidates from a blood sample in an array with single-occupancy distribution by conventional, brightfield microscopy. We demonstrated that our technique can identify samples from healthy patients and even distinguish between samples from patients with varying severity of cancer. Although the sensitivity is currently limited due to capture of a sub-population of target cells at the magnetic array, the specificity is high. Our negative-selection method avoids a potential bias that arises from other, positive-selection methods that use epitope- or size-based targeting of candidate cells. The strategy is thus applicable to all cancer types studied *via* blood sampling, including the detection of cells that do not express the well-known epitopes such as EpCAM or cytokeratin, or that display a size range that overlaps with normal white blood cells.

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