CLINICAL SIGNIFICANCE OF Viable-ENRICHED CIRCULATING Tumor CELLS WITH A FLEXIBLE MICRO SPRING ARRAY

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ABSTRACT
Circulating tumor cells (CTCs) in peripheral blood are implicated in the spread of cancer by metastasis. CTCs are associated with worse survival and have been demonstrated as a prognostic biomarker in various cancer types. We have developed a flexible micro spring array (FMSA) device for antigen-independent viable-enrichment of CTCs from whole blood based on cell size and deformability. CTCs were enriched from twenty blood samples obtained from advanced non-small cell lung cancer (NSCLC) patients and quantified through immunocytochemical detection. CTC detection was correlated to a significant decrease in patient survival demonstrating the clinical relevance of FMSA-enriched CTCs in NSCLC.

KEYWORDS: Circulating Tumor Cell, Enrichment, Microfiltration, Lung Cancer

INTRODUCTION
The spread of malignant tumors by metastasis accounts for the majority of cancer-related deaths [1]. The process of metastasis in carcinomas involves an epithelial-mesenchymal transition (EMT) of tumor cells which enhances their ability to migrate and penetrate into the peripheral circulatory system. Access to circulation facilitates their travel to colonize distant locations and form metastatic tumors. When these cells are present in peripheral blood they are referred to as CTCs. The detection of CTCs is associated with poor prognosis. CTCs captured through immunomagnetic enrichment targeting the epithelial antigen EpCAM have been established as a prognostic biomarker predictive of patient survival in breast [2], prostate [3] and colorectal cancer [4].

We have developed the FMSA microdevice for antigen-independent enrichment of viable CTCs. The FMSA consists of flexible spring elements patterned from parylene-C polymer that act as filtration structures to separate cells at the micro-scale. The device has been demonstrated to achieve 90% capture efficiency, higher than $10^4$ enrichment against leukocytes, and better than 80% viability from 7.5 mL blood samples in less than 10 minutes [5]. Since the method of FMSA CTC enrichment discriminates between cells based on their size and deformability it is presumed that the obtained CTC subpopulation may be distinct from those cells captured with antibodies for EpCAM. This designates a need to establish the clinical relevance of size/deforrnability enriched CTCs through clinical studies correlated to patient outcomes. Here we describe the application of the FMSA system to blood samples obtained from advanced non-small cell lung cancer (NSCLC) patients to demonstrate the clinical potential of FMSA-enriched CTCs.

EXPERIMENTAL
The FMSA was microfabricated as previously described [5] and clamped within a testing assembly consisting of polydimethylsiloxane (PDMS) sealing o-rings and a plastic housing (Figure 1A). 7.5 mL blood samples were drawn from NSCLC patients through peripheral venipuncture or from a central line into EDTA tubes and processed within 24 hours. Samples were passed directly through the FMSA assembly without fixation, preservation, or any prior processing to enable viable-enrichment. Filtration driving pressure was regulated to 1 inch water column since low pressure viable-enrichment allows cells to retain their morphology (Figure 1B) as opposed to the loss of viability and profile that can occur with unregulated pressures (Figure 1C).
RESULTS AND DISCUSSION

After viable-enrichment captured CTCs were detected with an immunocytochemical assay for the presence of epithelial antigens cytokeratins 8/18/19, the absence of the leukocyte common antigen CD45, and positive cell nucleus staining (DAPI). CTCs enriched from NSCLC patient samples were observed to have a wide range of sizes and morphologies as can be seen in Figure 2. The average CTC diameter (mean ± SD) was 27.5 ± 11.8 µm with a range of 10.8 - 77.8 µm. The presence of massive multinucleated CTCs accounted for the largest measured diameters. No cells with the CTC phenotype were detected in 10 processed negative control samples obtained from healthy donors. The positive detection limit was therefore set at the level of 1 CTC.

Figure 2: Immunocytochemical detection of FMSA-enriched CTCs from NSCLC patient samples. Composite fluorescence images include: cytokeratins 8/18/19 (green), CD45 (magenta) and DAPI (blue). Scale bar is 30 µm.
The FMSA device enriched at least 1 CTC in 12 out of 20 NSCLC patient samples (Figure 3A). The mean was 17.9 CTCs and the median was 5 CTCs per 7.5 mL blood sample. Probability of overall survival was calculated based on CTC analysis and is plotted in Figure 3B. The group of patients with ≥ 1 CTC detected from a 7.5 mL blood sample had a median survival of 124 days, while the group that did not have detectable CTCs had a median survival of 349 days. The difference in survival probability was statistically significant according to the Log Rank test (p < 0.05). This demonstrates the clinical relevance of size/deformability based viable-enriched CTCs with the FMSA device in NSCLC, and indicates their potential utility as a prognostic biomarker.

![Figure 3: A: Counts of viable-enriched CTCs from NSCLC patient samples. B: Kaplan-Meier estimates of overall survival probability for patients with no detectable CTCs (n=8) and with ≥ 1 CTC (n=12).](image)

**CONCLUSION**

The FMSA could successfully enrich and detect CTCs from NSCLC patient samples. Patients with at least 1 CTC detected in a 7.5 mL blood sample were found to have a statistically significant decrease in overall survival compared to patients with no detectable CTCs. This result indicates the clinical significance of FMSA-enriched CTCs in NSCLC and their potential to be applied to further clinical study.

**ACKNOWLEDGEMENTS**

We thank the Penn State Materials Research Institute and Nanofabrication Laboratory. This work was supported by a grant from the Pennsylvania Department of Health using Tobacco CURE Funds and by the National Cancer Institute of the National Institutes of Health award number DP2CA174508.

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