Lab-on-a-Chip Technologies for Oral-Based Cancer Screening and Diagnostics

Capabilities, Issues, and Prospects

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ABSTRACT: The design of a microfluidic lab-on-a-chip system for point-of-care cancer screening and diagnosis of oral squamous cell carcinoma (OSCC) is presented. The chip is based on determining a ~30-gene transcription profile in cancer cells isolated from oral fluid samples. Microfluidic cell sorting using magnetic beads functionalized with an antibody against cancer-specific cell-surface antigens (e.g., epithelial cell adhesion molecule [EpCAM]) is described. A comprehensive cancer diagnostics chip will integrate microfluidic components for cell lysis, nucleic acid extraction, and amplification and detection of a panel of mRNA isolated from a subpopulation of cancer cells contained in a clinical specimen.

KEYWORDS: cancer diagnostics; oral squamous cell carcinoma (OSCC); microfluidics; lab-on-a-chip; EpCAM

INTRODUCTION

Oral squamous cell carcinoma (OSCC), constituting 40% of all head and neck cancers, offers both a compelling opportunity and illustrative case study for the development of new point-of-care cancer diagnostics technology. Oral cancer as a target for new cancer screening modalities is a good choice because early detection methods are sorely lacking. Despite advances in diagnostics...
and therapy, the 5-year survival rate for OSCC patients remains at about 50%. Early detection of OSCC could greatly reduce morbidity by fostering more timely initiations of therapy and patient monitoring, and also would help avoid inappropriately aggressive surgical treatments that result in severe disfigurement. Moreover, oral fluid samples from OSCC patients collected by noninvasive methods are found to contain precancerous (dysplastic) and cancerous cells that (1) express specific cancer markers that serve as molecular targets for sensitive and specific detection, and (2) are amenable to more elaborate analysis (typing and staging) using gene expression profiling.

There is an urgent need for new technologies to enable inexpensive, convenient, and rapid cancer screening and diagnostics. Cancer tests that could be employed at the point-of-care, for example, doctors’ and dentists’ offices, and operated without extensive training or expertise are of special interest. Lab-on-a-chip microfluidics3–5—the miniaturization of fluidic networks for chemical and biochemical processing and analysis—offers a means for mass-produced, low-cost, single-use (disposable) devices for cancer screening and diagnostics, providing easily interpreted test results in a time frame of 10 to 60 min. Ideally, these lab-on-a-chip cancer diagnostics systems would use 10 to 1,000 μL of various types of clinical specimens including oral fluids, whole blood, serum, or urine, collected by minimally invasive methods, as well as samples, such as tissue biopsies, intraductal breast fluid, bronchial lavages, and lung aspirations. In general, the anticipated benefits of microfluidics for clinical diagnostics derive from the use of small sample volumes, automated operation, short processing times, and near real-time reporting of results, reduced reagent consumption, reproducibility and consistency, reduced exposure to hazardous materials and infectious agents, minimal risk of sample contamination, convenient disposal, and low cost.

In the last decade, a diverse array of microfluidic components and systems have been developed for immunoassays and include cell sorting, detection and counting, lysis, nucleic acid and protein isolation and amplification, and detection and quantification of nucleic acids and proteins.3–5 Lab-on-a-chip devices for detection of infectious agents and toxins are becoming well established. The microfluidic technology developed for pathogen detection can be adapted and extended for the more difficult task of cancer screening and diagnostics. In the simplest approach, an automated immunoassay of a single cancer marker or a panel of cancer markers can be implemented on a credit card–sized microfluidic cassette for point-of-care cancer screening. More robust and detailed tests can be realized by quantifying a panel of 10 to 30 mRNA or proteins for determining a cancer-specific gene transcription or expression profile. To assess a gene transcription profile, microfluidics systems need to include components for cell sorting to enrich the sample in cancer cells, cell lysis, nucleic acid isolation, multiplex reverse transcriptase-polymerase chain reaction (RT-PCR) or other analogous amplification techniques, and multiplex detection and quantification of the gene transcripts. Newly developed
bio-barcode assays for multiplex detection of proteins\textsuperscript{6} and gene transcripts\textsuperscript{7} offer an alternative approach for nonenzymatic multiplex amplification and detection of both nucleic acids and proteins.

We assess the feasibility of a lab-on-a-chip molecular diagnostics system for OSCC screening and diagnostics using oral fluid samples. Processing steps for cancer diagnosis are identified. Supporting data from benchtop studies demonstrating methods for isolating cancer cells from oral fluids, and gene expression analysis identifying an OSCC-related transcription profile, serve as the basis of the microfluidic cancer diagnostics system. We present a design for a lab-on-a-chip system that tests for a cancer-related gene transcription signature by assaying a panel of mRNA extracted from precancer or cancer cells isolated from an oral fluid sample.

\textbf{Figure 1} depicts a flow process for a cancer diagnostics protocol whereby a cancer-specific gene expression profile is determined. The cancer diagnostic process comprises an initial step to remove lymphocytes that interfere with subsequent steps for isolating cancer cells from the sample. The sample is depleted of lymphocytes by immunoseparation using magnetic beads coated with anti-CD45 antibody, which binds to lymphocytes. Next, the cancer cells are sorted from the sample using magnetic beads coated with anti-epithelial cell adhesion molecule (EpCAM) antibody. EpCAM is a cell membrane glycoprotein that is aberrantly expressed on the surface of cancerous epithelial cells associated with OSCC. The separated cancer cells can be detected and (optionally) counted. The detection of EpCAM-expressing cells in the sample serves as the first screening test for cancer. The separated cancer cells are then subjected to a thermal and/or chemical lysis step, and the mRNA are isolated from the lysate using solid-phase extraction or by hybridization with magnetic beads coated with mRNA-specific oligonucleotides. Multiplex mRNA amplification by RT-PCR, linear amplification, or a bio-barcode technique is followed by detection of labeled cDNA, aRNA, or bio-barcodes using either fluorescence with fiber optic sensors or electrochemical sensors.

\section*{SUPPORTING STUDIES}

Cancer cells are isolated from the sample by immunoseparation using paramagnetic beads coated with antibodies that bind to cell membrane proteins specific to cell types (including lymphocytes and cancer cells) making up the heterogeneous sample. The relevant findings may be summarized as follows:

1. Western blots with Ber-EP4 (monoclonal antibody to EpCAM) indicate that EpCAM is expressed only in OSCC cells and is not detectable in normal cells or fibroblasts. Our findings are consistent with a growing body of literature documenting high EpCAM expression in various cancer cells of epithelial origin.\textsuperscript{8}
FIGURE 1. Sample processing steps for isolating cancer cells from saliva and assaying a panel of mRNAs.

2. Magnetic beads functionalized with Ber-EP4 separate cancer cells from a suspension containing known quantities of labeled cancer cells (from various cell lines) and normal cells. In a negative control, magnetic beads functionalized with anti-IgG failed to bind to either cancer or normal cells. Figure 2 shows a cancer cell tagged with 4.5-μm diameter magnetic beads functionalized with anti-EpCAM antibody.
FIGURE 2. OSCC cancer cell bound with four 4.5-μm diameter superparamagnetic beads (Dynal, Invitrogen, Carlsbad, CA, USA).

3. Magnetic beads conjugated with antibodies to CD45 separated lymphocytes from a mixture containing $2 \times 10^7$ lymphocytes and various quantities of stained cancer cells. Subsequently, the cancer cells were isolated (with greater than 80% efficiency) using magnetic beads functionalized with Ber-EP4.

4. Subsequent to the removal of lymphocytes with magnetic beads functionalized with antibodies to CD45, magnetic beads functionalized with Ber-EP4 isolated more than 9,000 cells/mL from unstimulated whole saliva from T4 patients, more than 1,000 cells/mL from T1 patients, and less than 15 cells/mL from healthy patients (Fig. 3). T refers to tumor stage of the tumor node metastasis (TNM) classification system, and 1–4 denotes tumor size (1 smallest, 4 largest). The epithelial origin of the isolated cells was demonstrated by staining them with pan-cytokeratin. The isolated cells were further identified as OSCC cells by labeling with antibody to HSP-47 (clone M10.1061). HSP-47 was shown to be singularly expressed on OSCC cells.

5. HSP-47 has been identified as another protein that is uniquely expressed on OSCC cell membranes and that can be used for labeling and discriminating isolated cells.9

6. A 25-gene transcription signature for OSCC can classify normal and OSCC specimens. This 25-gene predictor was 96% accurate on cross-validation, averaging 87% accuracy using three independent validation tests.10
FIGURE 3. Isolation of tumor cells in OSCC patient saliva. A process using negative selection first with magnetic beads bound to the antibody CD45 (the major lymphocyte marker) followed by positive selection with magnetic beads functionalized with Ber-EP4 antibodies recovered the tumors. Total tumor cells isolated from each tumor are shown. Essentially no cells were isolated from normal patients’ saliva. These numbers of cells should be sufficient for obtaining enough RNA for complete analysis.

MICROFLUIDIC IMPLEMENTATIONS AND DESIGN APPROACHES

FIGURE 4 shows a microfluidic device for continuous sorting of cells using the principle of magnetic field flow fractionation (MFFF).\textsuperscript{11} A mixed population of cells (cancerous and noncancerous) are incubated with 4.5-\textmu m diameter superparamagnetic beads (Dynel Cellection\textsuperscript{TM} Epithelial Enrich, Invitrogen, Carlsbad, CA, USA) coated with anti-EpCAM antibody to selectively tag the target cancer cells with magnetic beads. The incubated sample is injected into a flow channel of a polycarbonate chip, and hydrodynamically focused with a surrounding sheath of flowing buffer. Both sample and buffer are propelled by programmed syringe pumps. To allow flow visualization, dyes are added to the fluids. The sample stream remained confined axially within the buffer sheath along the chamber’s entire length (Fig. 4A). The sample stream is subjected to the field of an external permanent magnet positioned as indicated. Figure 4B is a histogram of the transverse distances (y) traveled by the bead–OSCC cell complexes, unlabeled cells, and free beads. The measurements were taken a short distance downstream of the injection point (dashed circle in Fig. 4A) with or without the external magnetic field. In the absence of a magnetic field, the cells and beads remained in the core of the flow and were kept separated from
FIGURE 4. (A) MFFF device for separating magnetic bead-bound cells from unbound cells and unbound beads. Cancer cells (expressing the surface protein EpCAM) and normal cells (no EpCAM) are mixed with 4.5-μm diameter superparamagnetic beads (Dynal Collection Epithelial Enrich, Invitrogen) and injected into a flow channel of a polycarbonate microfluidic chip. The sample is hydrodynamically focused with a sheath of buffer solution surrounding the sample stream. (B) Histogram showing deviation of flow path due to the applied magnetic field. With no applied field, the beads, unbound cells, and bead–cell complexes follow an unimpeded axial trajectory in the flow channel. Application of a magnetic field with an external permanent magnet causes the beads and bead–cell complexes to deviate from the axial flow and follow a characteristic trajectory, resulting in separation of beads, unbound cells, and bead–cell complexes.

the chamber walls by a surrounding buffer. When a magnetic field was applied, the magnetic beads and labeled cells diverted from the sample stream with the free beads moving faster and at a sharper angle with respect to the axis than the labeled cells (FIG. 4B). The unlabeled cells maintained their axial trajectory. Thus, bead–cell complexes can be separated (and collected) according to their distinct trajectories resulting from application of magnetic field.

FIGURE 5 depicts a schematic plan view for a microfluidic cassette that performs the steps outlined in FIGURE 1. The crucial function of isolating cancer cells from a heterogeneous clinical specimen, such as oral fluid by microfluidic immunoseparation with magnetic beads, appears feasible. Microfluidic components for lysis, nucleic acid isolation, multiplex RT-PCR and detection,
FIGURE 5. Plan view schematic of a comprehensive cancer diagnostics lab-on-a-chip integrating microfluidic components for lymphocyte depletion, cancer cell isolation and lysis, mRNA isolation, multiplex amplification, and detection of a panel of mRNA.

or analogous processes, such as bio-barcode signal amplification, have been demonstrated by numerous groups—see, for example, Ref. 12. The outstanding challenge now is to seamlessly integrate the appropriate microfluidic components into a comprehensive, cost-effective lab-on-a-chip system for automated
operation that provides easily interpreted, statistically significant cancer diagnostics data in a timely manner.

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