**Design of a microfluidics device to rapidly genetically analyze bronchial epithelial cells for EGFR mutation**

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**INTRODUCTION**

 Lung cancer is a growing epidemic in America. In 2012 alone, an estimate 226,000 people were diagnosed with lung or bronchial cancer, while another 160,000 died [1]. In addition, lung cancer is the second most commonly diagnosed cancer for men and women, just behind prostate and breast cancers, respectively. More people, of both sexes, died from lung cancer in 2012 than any other type of cancer. In fact, lung cancer killed more people than prostate, breast, pancreas, and colon cancers put together (Figure 1).



**Figure 1: Lung cancer deaths compared to death from other types of cancer [2].**

 Currently, the only definitive detection methods for lung cancer are chest x-rays, computed tomography (CT), and surgical biopsy of possibly cancerous tissue. However, lung cancer is first suspected based on the symptoms of chest pain, blood in expectorate, shortness of breath, and weight loss [3]. Currently, the American Society of Clinical Oncologists suggests yearly screenings of low-dose computed tomography for at risk individuals. The Society does not recommend, however, the screening of individuals who may develop lung cancer from working with industrial carcinogens (like asbestos) or individuals who have had significant exposure to secondhand smoke over the course of their lifetime. In 2012, these “never smokers” represented 10-20% of all lung cancer diagnoses [4].

 No effective screening method for lung cancer exists apart from CT. CT can give detailed anatomical information, but often lacks in the ability to differentiate between malignant and benign cancerous lesions [5]. Chest x-rays are not sensitive enough to pick up on the earlier stages of lung cancer [5]. In addition, both these methods are impractical for use as annual screening methods over a number of years due to the high dosage of radiation that can be incurred on the patient. As a result, lung cancer is not often definitively diagnosed until histology can be performed on tissue from a surgical biopsy. However, by the time a surgical biopsy is performed, lung cancer is already suspected and may be at an advanced stage. This has led researchers to look for alternative ways to diagnose lung cancer using molecular biology and non-invasive sample collection methods.

*Epidermal growth factor recpetors*

 Epidermal growth factor receptor (EGFR) is a protein found on cell membranes. Upon interaction with growth factor ligands, the receptor activates several cellular signaling cascades, including ones for cell division [6]. It should be no surprise then that mutations affecting the regulation of EGFRs have been found to be associated with cancer [7]. Mutations often occur on exons 18-21 of the EGFR gene; this string of code is associated with the part of an EGFR that increases kinase activity. Commons mutations are a deletion in exon 19 and a point mutation at exon 21. These mutations are also extremely common in “never smokers” who still develop lung cancer [8].

 EGFRs are present in many cells, including bronchial epithelial cells where they have been implicated in damage repair and mucus secretion [9]. Genetic analysis of these cells taken from the airway can provide information regarding the presence of EGFR mutations.

*Collection and enrichment of sputum*

 A sputum sample for genetic analysis can be obtained non-invasively [10] but requires enrichment before genetic examination [11, 12]. After enrichment, each sample contains enough material to undergo gene chip analysis as demonstrated by Jiang et. al [5]. Since the device presented here will require approximately the same amount of genetic material as needed for a gene chip, a post-enrichment sputum sample can be used for analysis.

*Detection of EGFR for lung cancer diagnosis*

Extensive research has already been completed on using genetic material to determine the presence of mutated EGFRs [13]. In addition, The most commonly used method is direct sequencing, in which DNA containing the mutated exon 19 are amplified and detected using gel electrophoresis. However, these methods usually require large amounts of DNA in the form of samples from invasive procedures, like tissue from surgical biopsy or pleural effusion from thoracentesis. Some procedures, like direct sequencing, require that at least 20% of the cells examined must have the EGFR mutation for the test to register as positive. Since the range of actual tumor cells containing the mutation in a histological sample can vary from 5 to 100% [13], tests like direct sequencing can miss important diagnostic information.

 Another method for the detection of mutation-bearing genetic sequences is denaturing high performance liquid chromatography (dHPLC). Sueoka et al. [14] determined that using dHPLC for analysis was faster (five hours for dHPLC versus eighteen hours for direct sequencing) and cheaper (direct sequencing was eight times more expensive). In addition, dHPLC provided more accurate results than direct sequencing. Currently, HPLC (though not dHPLC) has shown promise as an analysis method that can be miniaturized onto a microfluidics device [15]. Other screening methods for the EGFR mutation include massively parallel sequencing analysis (or second generation sequencing) [16] and high resolution melting [17]. However, all of these analysis methods require amplification of the genetic material, a time-consuming process, and the latter two methods have yet to be studied for miniaturization onto microfluidics devices. In addition, the need for an amplification step also requires that the sample already contains high amounts of mutation-bearing cells; these samples will have to come from invasive biopsy procedures like those mentioned above. Another, though much less commonly used, method for EGFR detection is the use of immunochemistry to bind directly to the mutated receptors themselves [18]. However, specific recombinant antibodies are needed to prevent non-specific binding with wild-type EGF receptors and other proteins [19].

 The goals of the device presented here are to create a fast, reliable, inexpensive, and non-invasive method to screen at-risk individuals with lung cancer. Since the device is miniaturized onto a microfluidics device, analysis time, reagent cost, and required sample volume will be much less compared to current sequencing technologies. Since the device will require only a non-invasive sputum sample, it can be used feasibly on at-risk populations instead of those already presenting symptoms of lung cancer. In addition, the device will be able to detect EGFR mutations commonly present in non-smoking individuals.

*Benefits of using a microfluidics device*

 Microfluidic devices, and specifically lab-on-a-chip devices like the one proposed here, incur several advantages over standard macroscale benchtop procedures simply due to their size [20]. The most often cited benefit is that smaller sample sizes are needed. Microfluidic analysis is usually inexpensive because the amount of possibly expensive reagents used is drastically reduced. In the realm of clinical diagnostics, this translates to a reduction in sample size. This allows for less invasive procedures, like sputum procurement, to be used diagnostically instead of surgical biopsy.

 Because volumes are so small, diffusion is often the major drive for mixing samples. This decreases the amount of time required for reactions or other processes to take place. With smaller volumes and shorter data collection times, assays are more sensitive and results are more accurate.

**METHODS**

The methods used by the device for detection of a mutated mRNA sequence for EGFR are presented below. The main focus is on detection of the mutated mRNA; mRNA is used instead of the original mutated DNA as it demonstrates active translation of the affected receptor by the cell. After the sample of bronchial endothelial cells in PBS is introduced into the device, the cells undergo electrochemical lysis. The mRNA contained in the cells is then purified using a silica bead microcolumn. The target mutated mRNA in the elution are then hybridized with complementary ssDNA (single-stranded DNA), creating dsDNA (double-stranded DNA). The presence of either unhybridized ssDNA or hybridized dsDNA changes the color of a colloidal gold nanoparticle solution, based on electrostatic interactions. A blue color, which can be determined visibly, represents the presence of the mutation while a pink color is represents a negative result.

*Electrochemical Cell Lysis*

 Di Carlo et al. [21] have designed a novel reagent-free cell lysis microfluidics device. The device uses tiny platinum electrodes to generate –OH and H+ ions in solution. The introduction of a high amount of hydroxide ions will destroy cell membranes (Figure 2), allowing for the release of genetic material. A filter (Figure 3) separates the cathode, which produces hydroxide ions, and the anode, which produces hydrogen ions. The purpose of the filter is to keep the introduced cells in close proximity to the cathode until lysis occurs; as a result, the filter size must be less than 10 microns, as this is the approximate diameter of bronchial epithelial cells [22]. The hydroxide-containing lysate will be free to move pass the filter and combine with the hydrogen ions being produced at the anode, creating a neutral solution for use in the next step. This process has been shown to work with red blood cells, HeLa, and Chinese Hamster Ovary cells, so testing will be required to prove effectiveness at lysing human epithelial bronchial cells. It can be hypothesized that since the HeLa cell line comes from human cervical epithelial cells, the process that works for HeLa may also work for epithelial bronchial cells.



**Figure 2: The reaction responsible for lysing cell membranes at the cathode [21].**

**Figure 3: A schematic and micrograph of the filter that separates the anode from the cathode. Only lysed material can pass through [21].**

*mRNA microcolumn Purification*

 Poeckh et. al [23] present a miniaturized silica bead microfluidics device for purifying DNA and RNA from cell lysate. Their approach to the design mimics macroscale purification methods. The cell lysate is pushed through a bed of silica beads in a solution with a high ionic strength, like sodium chloride or guanidinium thiocyanate (GuSCN). The solution causes DNA and RNA to bind to the surface of the silica beads. A subsequent wash through of a neutral solution removes all other non-bonded debris. The DNA or RNA is then released from the silica beads with an elution buffer and pushed out the column. Macroscale purification makes use of centrifugation to pull the lysate, wash, and elution solutions through the column; however, this is not feasible for a microfluidics device. Instead, Poeckh et. al found that using a pressure gradient to drive the fluid flow in the device was sufficient.

 Poeckh et. al also found the elution times for RNA and DNA at a flow rate of 100 µL/hour with 6M GuSCN at a pH of 8; most of the RNA elutes with the first 2 µL of solution, while DNA elutes between 3 to 6 µL. The solution used for the elution step was molecular biology grade water.

 For the microfluidics device presented here, the mRNA purification step represents the most complicated part of the setup, design-wise. Separate inlets would need to be addressed for the introduction of GuSCN, wash buffer, and MBG water, as well as separate outlets for the removal of lysate and elution. However, the volume of the reagents required are very small and could conceivably come pre-packaged in the device.

*Hybridization of mRNA to complementary ssDNA*

 The next step is to promote the hybridization of the purified mRNA to complementary ssDNA bearing the mutation. The sDNA will have to be synthesized via RT-PCR from mRNA known to carry the mutation [24]; this step will be carried out beforehand and separately from the rest of the procedures described here, and will not involve the device. In the device, the purified mRNA and ssDNA will be combined in a new chamber containing 10 mM PBS and 0.3 NaCl. The hybridization reaction will take place over the course of five minutes at room temperature [25].

 Li et. al [25] have also found a way to prevent the detection of mismatched hybrids (or ds’DNA). Dissociation of the hybrid occurs after several minutes, by dissociation of ds’DNA occurs more quickly than dsDNA. Waiting an extra two minutes after the hybridization reaction allows for dissociation of the ds’DNA while the dsDNA remains bound.

 If the mutated mRNA was present in the purified sample, dsDNA will be created during the hybridization reaction. If no mutated mRNA was present, the other genetic material present in the elution will fail to bind to the complementary ssDNA. As a result, a sample lacking the mutated mRNA will produce only ssDNA after the hybridization step.

*Addition of colloidal gold solution*

 The transduction method of the device relies solely on whether ssDNA or the hybridized dsDNA is present in the sample. After hybridization and dissociation of any ds’DNA, the colloidal gold nanoparticle solution is added to the chamber. As Li et. al [25] have discovered, ssDNA and dsDNA have different electrostatic interactions with gold nanoparticles. The ssDNA can present a side of unbound nucleotides; this causes the ssDNA molecules to stick electrostatically to the nanoparticles. In contrast, the dsDNA is encased in a negatively charged phosphate-sugar backbone. As a result, few dsDNA molecules will attach electrostatically to the nanoparticles.

The addition of a salt solution to a gold nanoparticle colloid causes the particles to aggregate; as the color of gold colloidal solutions is dependent on particle size, this aggregation changes the color of the solution. However, ssDNA electrostatically attracted to the gold nanoparticles act to negate the attractive forces presented by the salt solution. As a result, a colloidal gold solution with ssDNA will not change color upon the addition of salt solution. However, no such electrostatic barriers exist for the particles if only dsDNA is present in the sample, and the solution will change color upon the addition of salt solution (Figure 4). This change in color which is visible in 5 µL down to 100 fmol of DNA present in the sample can be used to detect the presence of the EGFR mutation without the use of optical instrumentation.



**Figure 4: Color change associated with electrostatic interactions of gold nanoparticles with DNA [25].**

**TESTING**

 The steps taken by the device to diagnose the presence of the EGFR mutation are inherently complex. Thus, testing of the device will begin with an analysis of each step individually.

*Electrochemical cell lysis*

 The question of whether the electrochemical method of lysing cells is effective can be confirmed via microscopic visualization. Di Carlo et. al [21] stained their HeLa cells with calcein to confirm cell lysis in real time using a fluorescent microscope.

 The next question is whether the lysed cells present in the sample can contribute a sufficient quantity of genetic material to the analyte. The amount of EGFR mutated mRNA can be quantified by running qPCR on the lysate. The colloidal gold solution requires at least 100 fmol of genetic material to be present to allow for visualization of the color change. The quantity given by qPCR can also be used to determine the amount of complementary ssDNA that needs to be added in the hybridization step.

*mRNA microcolumn purification*

 The purification step needs to be tested to ensure that only and all genetic material from the lysate passes through the silica bead column. Lysate known to contain the mutated mRNA will be passed through and analysis of the elution will be performed using qPCR. This will also quantify the amount of mutated mRNA and ensure that the concentration has not decreased below 100 fmol before hybridization.

*Hybridization of mRNA to complementary ssDNA*

 Northern blotting can be used to verify and optimize the hybridization the mRNA and DNA under microfluidic conditions.

The main goal in testing the hybridization step is to optimize the amount of ssDNA that needs to be added. Microarrays of oligonucleotides complementary to the ssDNA can be used. By using a microarray with a number of oligonucleotides comparable to the concentration of mutated mRNA present after elution, different concentrations of fluorescently-labelled ssDNA can be introduced to the microarrays. The concentrations of the ssDNA added will be increased until there is no longer a measurable increase in fluorescence. The highest concentration up to this point will be used for the microdevice. Goff et. al [26] used a similar method of optimization for detection of miRNA.

*Addition of colloidal gold solution*

The color change associated with the gold colloid will have to be verified first with no genetic material present. Next, different concentrations of the colloid will be added to the expected sample concentrations of ssDNA and dsDNA, as determined above. The concentration that provides the most definite color change will be used.

Once testing of all the separate components is completed, the sample will be run from start to finish with all the concentrations and volumes expected to be used in the microfluidics device, and any overall design flaws or changes can be made to the experimental protocols. Next, based on the experimental protocols, the actual design of the device can begin. Different designs will be fabricated and tested with sputum samples known to contain the EGFR receptor mutation, and samples that do not.

When a design has been chosen and optimized, clinical testing on lung cancer patients can begin. The device will first be used alongside more conventional methods of detecting lung cancer. Finally, a long-term study will be performed in which individuals testing positive on the device for the EGFR mutation will be monitored for the possible development of lung cancer.

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