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Lab-On-A-Chip for Oral Cancer Screening and Diagnosis

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Abstract
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Keywords
oral cancer, lab-on-a-chip, microfluidics, biomarkers, screening

Disciplines
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BASIC SCIENCE REVIEW

Robert L. Ferris, MD, PhD, *Section Editor*

LAB-ON-A-CHIP FOR ORAL CANCER SCREENING AND DIAGNOSIS

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Abstract: Oral squamous cell carcinoma (OSCC) is a disfiguring and deadly cancer. Despite advances in therapy, many patients continue to face a poor prognosis. Early detection is an important factor in determining the survival of patients with OSCC. No accurate, cost-efficient, and reproducible method exists to screen patients for OSCC. As a result, many patients are diagnosed at advanced stages of the disease. Early detection would identify patients, facilitating timely treatment and close monitoring. Mass screening requires a rapid oral cancer diagnostic test that can be used in a clinical setting. Current diagnostic techniques for OSCC require modern laboratory facilities, sophisticated equipment, and elaborate and lengthy processing by skilled personnel. The lab-on-chip technology holds the promise of replacing these techniques with miniaturized, integrated, automated, inexpensive diagnostic devices. This article describes lab-on-chip devices for biomarker-based identification of oral cancer. Similar methods can be employed for the screening of other types of cancers. ©2007 Wiley Periodicals, Inc. *Head Neck* **00:** 000–000, 2007

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Head and neck cancers are the sixth most common cancer worldwide and are associated with low survival and high morbidity.[1] Cancers of the oral cavity account for 40% of head and neck cancers and include squamous cell carcinomas of the tongue, floor of the mouth, buccal mucosa, lips, hard and soft palate, and gums.[2,3] Despite therapeutic and diagnostic advances, the 5-year survival rate for oral squamous cell carcinoma (OSCC) remains at about 50%.[2–4] In addition, aggressive treatment of OSCC cancer is controversial since it can lead to severe disfigurement and morbidity.[5] As a result, many patients with OSCC cancers are either over- or under-treated with significant personal and socio-economic impact.

One of the major factors accounting for the poor outcome of patients with OSCC is that a great proportion of oral cancers are diagnosed at advanced stages and, therefore, treated late. Early detection of pre-malignant or oral cancer

lesions will greatly reduce morbidity associated with late disease treatment and improve overall patient survival. For example, early detection could lead to frequent patient monitoring, dietary changes, counseling for cessation of smoking and drinking, preventative drug administration, and lesion removal. Indeed, early diagnosis and treatment of OSCC lead to a mean survival rate of over 80% and a good life quality after treatment.[6] Thus, screening methods are needed to detect OSCC cancers.

For most practitioners, visual screening of patients for precancerous or cancerous lesions is the most common screening method. Recent studies in India have reported that oral visual screening can reduce mortality in high risks individuals (tobacco and alcohol users[7–9]). However, visual screening is limited in that it only identifies whether a lesion is present. Visual screening cannot, for example, determine the progression of a stage I or stage II tumor to stages III/IV or distinguish which leukoplakia or dyplastic lesions will progress to carcinoma. Therefore, a screening system is needed that will identify which pre-cancer lesions will progress to cancer.

Currently, clinical examination and histopathological studies are the standard diagnostic method used to ascertain whether biopsied material is a precancerous or cancerous lesion.[10,11] The reliance on biopsies has several drawbacks: (1) biopsies are invasive procedures and, depending on the site, often involve surgical techniques and anesthesia; (2) small lesions may not provide sufficient material for accurate diagnosis; (3) biopsies from sites within a large lesion may not reflect the entire histopathological aspects of the lesion; (4) biopsies are inherently subjective in nature; and (5) sensitivity and specificity of biopsies are limited, increasing the potential for flawed diagnosis and an inappropriate therapeutic approach. Thus, additional methodologies are necessary to detect pre-malignant and malignant oral cancer lesions.

Non-invasive detection of pre-malignant and malignant oral cancer cells requires easy access to the site where these cancers typically arise and a readily available source of cancer cells. The oral cavity meets both of these criteria. Saliva from the oral cavity, because of its cellular composition, accessibility, and inexpensive and non-invasive methods of collection, is ideal as a diagnostic medium for oral cancer detection. Flow cytometry analysis of human saliva has demonstrated that saliva is composed of live and dead erythrocytes, leukocytes, and epithelial cells.[12] However, distinguishing normal, precancerous, and cancerous cells from the other cellular populations in saliva requires not only capture and enrichment of the precancerous and cancerous cells, but also cancer-specific biomarkers.

In this review, we will discuss combining biomarker detection and microfluidic lab-on-a-chip technology to develop a device for early screening of oral cancer. This microfluidic chip will accept saliva samples, will be operated by minimally trained personnel, and will provide a diagnostic answer in an automated and timely fashion. The detection of oral pre-cancer (dysplastic) and cancer cells within the chip will take advantage of membrane-associated cell proteins that are singularly expressed on the cell membranes of dysplastic and cancer cells and of the unique gene transcription profiles of cancer cells. As such, this system will provide a means for automated, rapid detection and molecular analysis of cancers in a miniaturized format suitable for use in the clinic and/or the operating room.

MOLECULAR TARGETS FOR THE ISOLATION OF ORAL CANCER CELLS

Analysis of exfoliated cells in saliva is the most direct method for screening oral pre-cancer and cancer lesions. Several approaches have been devised to detect oral precancer and cancer cells in saliva. Cytology is the most straightforward way and involves pathological evaluation of the exfoliated smear. Several variations or improvements to exfoliated cell cytology have been tested and include staining for the presence of micronuclei, detection of microsatellite instability (MSI) by polymerase chain reaction (PCR), fluorescence in situ hybridization (FISH) analysis, promoter hypermethylation, and mitochondrial DNA content.[13–18] While promising, these methods all involve analyzing the entire cellular population contained within salvia. Such an approach may have low sensitivity for tumor cell detection, especially pre-cancer cells.[19] To improve the sensitivity of pre-cancer and cancer detection, a method that enriches the pre-cancer and cancer cells from the cellular population of saliva would be preferable.

Expression of cancer-specific membrane proteins that could be used to discriminate cancer cells from normal cells and facilitate identification, capture, and enrichment of cancer cells would be ideal. For example, antibodies against cancer-specific membrane proteins conjugated

with labels such as fluorescent antibodies and quantum dots or beads (magnetic, metallic) can preferentially tag the cancerous cells in a sample. However, unique proteins expressed by the cancer cells must first be identified. Several recent reports have identified oral pre-cancer (dysplastic) and cancer cells express 2 membrane glycoproteins, which are either absent or minimally expressed in the membranes of normal cells. Interestingly, these same glycoproteins are being targeted for immunotherapeutic approaches for cancer treatment and therefore are excellent candidates for cancer diagnostics as well (Personal communication, Dr. Nick Glover, President, Viventia Biotech Inc.).[20]

Hsp-47, also known as colligin, is normally an endoplasmic reticulum (ER) resident protein with collagen binding properties. However, in several disease states including oral cancer development and progression, Hsp-47 modulates collagen production by locating to the cell surface membrane.[20] In oral cancers Hsp-47 is characterized as being present only on cancer cells where it is typically over expressed.[20,21] As such, Hsp-47 has been designated as a specific marker for oral cancer detection and as a potential biomarker for therapeutic targeting of oral cancers.[21] Like Hsp-47, EpCAM is also a cell surface protein found on cancer cells of the oral cavity.

EpCAM is a 40-kDa epithelial transmembrane glycoprotein that is strongly expressed in several epithelial cancers including colon, prostrate, head and neck, esophagus, lung, and breast.[22] EpCAM is not expressed by hepatocytes, thymic cortical epithelial cells, gastric parietal cells, myoepithelial cells, and many other non-epithelial cells.[23] EpCAM-negative squamous epithelial cells can express EpCAM upon transformation where the amount of EpCAM and the number of EpCAM expressing cells increases with the grade of dysplasia.[24] In more recent studies, EpCAM was found in >89% of all head and neck OSCC tumor specimens analyzed.[25] Furthermore, EpCAM has been detected in the majority, and expressed significantly higher, in primary squamous cell carcinoma of the tongue than in normal oral mucosa.[26] It was concluded from this study that EpCAM could be a molecular target for oral cancer. In agreement with this study, Chaubal et al[27] have demonstrated that EpCAM can be used as a tumor marker for the diagnosis of single tumor cells in patients with head and neck cancers. Thus, EpCAM appears to be an ideal cell surface protein for enriching oral pre-cancer and cancer cells from the saliva.

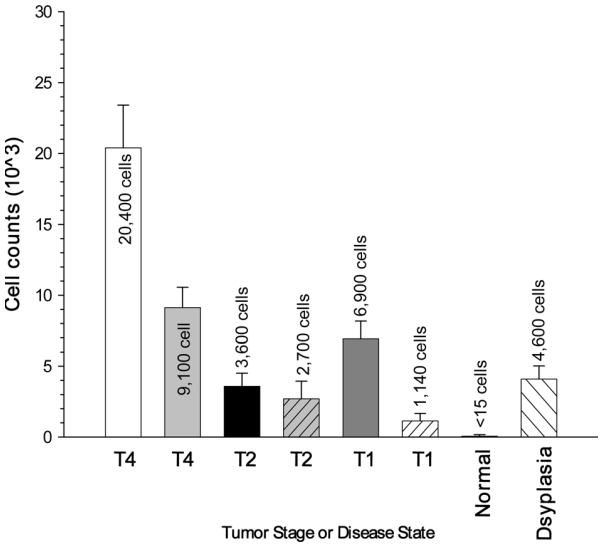

FIGURE 1. Isolation of tumor cells from OSCC patients' saliva. Using negative selection with magnetic beads bound to anti-lymphocyte antibodies, followed by positive selection with magnetic beads functionalized with antibodies to specific cell surface glycoproteins, we isolated the dysplastic and oral cancer cells. The total number of dysplastic or cancer cells isolated is depicted as a function of the stage (TNM) of the disease. Since the tumor cell yield depends on the size of tumor above or exposed to the mucosal surface (tumor cells below the mucosal surface do not slough off), there was only a weak correlation between the collected amount of cancer cells and tumor stage or size. Essentially, no cells were isolated from healthy patient saliva.

Additional studies, using magnetic beads coated with EpCAM antibodies, have shown that tumor cells can be efficiently isolated from saliva-like samples containing mononuclear cells. In initial work, saliva was replicated by mixing lymphocytes, obtained from healthy volunteers with cells from 3 human oral cancer cell lines at varying cell numbers in binding buffer. Magnetic beads functionalized with EpCAM antibodies were added and the number of tumor cells recovered was determined. The recovery yield was on average >80% in all cell lines and independent of the number of cancer cells.[19] In agreement with this initial study, we have determined that using antibodies specific for EpCAM we can isolate cancer cells in saliva obtained from patients with various OSCC TMN staged tumors and dysplastic lesions but not from healthy patients, with a false positive rate <0.05% (Figure 1). This is accomplished by removing interfering cells from saliva such as mononuclear cells and capturing the tumor cells with magnetic beads coated with antibodies specific to this membrane glycoprotein. Thus, the capture, enrichment, and counting of

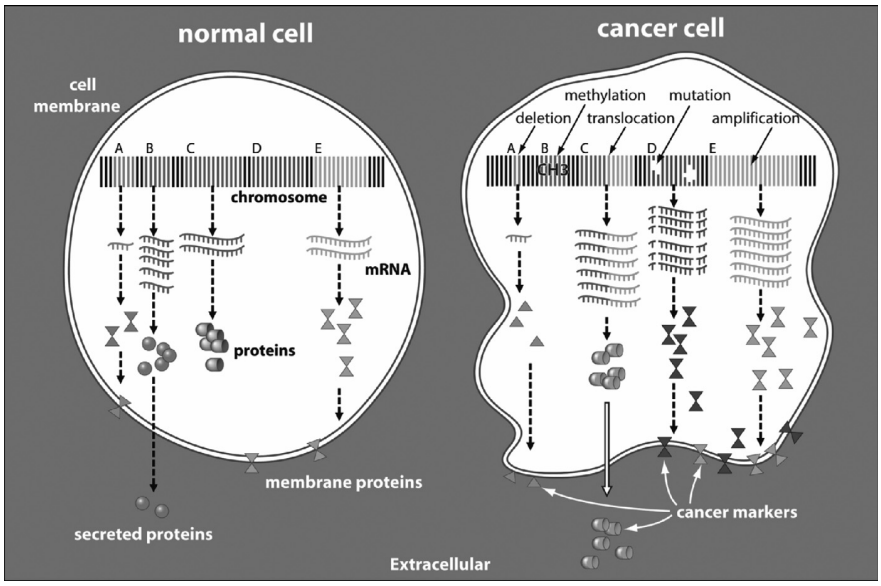

FIGURE 2. Illustration of differences between normal and cancer cells. Potential cancer biomarkers exemplified by genetic changes in the chromosomal DNA are illustrated in the cancer cell. Typical changes in the host DNA such as point mutations, deletions, translocations, amplifications, and methylations alter mRNA transcripts from these affected genes. These affected mRNA transcripts could be lost, mutated, or increased. As a result of the mRNA changes, cellular protein products from these affected genes are similarly altered. The altered proteins in the cancer cell are expressed intracellularly, on the cell surface, or secreted into the extracellular space at higher or lower levels compared to normal cells. Exploitation of specific changes that occur in the cancer cell's RNA or protein provides convenient targets to enrich the cancer cells from normal cells and other cell types. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the cancer cells isolated from a clinical sample using EpCAM can serve as an initial screen for cancer and, in itself, is a useful medical test. However, to gain additional information about the type and stage of the cancer, it is desirable to obtain the gene expression profile of the isolated cells.

GENE EXPRESSION PROFILING

Gene expression profiles have shown dramatic correlations with tumor development and progression, lymph node invasiveness, recurrent disease, and patient outcome when applied to biopsy samples.[28–31] Typically, genetic changes in cancer cells lead to altered gene expression patterns that can be identified long before the cancer phenotype has manifested (Figure 2). When compared with normal mucosa, the changes that occur in the cancer cell can be used as biomarkers. Several candidate genes associated with OSCC tumor progression such as p53, cyclin D1, and epidermal growth factor receptor (EGFR) gene have been identified.[32] However, to date, no single gene has shown sufficient diagnostic utility in OSCC. Thus, as in many other cancers, clinical diagnosis will require considering the combined influence of many genes.

Expression patterns of sets of genes have shown dramatic correlations with tumor behavior and patient outcome. Microarray analysis of several tumor types has demonstrated that global expression profiling can distinguish tumor cells from normal cells, as well as identify the class and subtype of cancer far superior to current histopathological diagnostic methods.[33–35] Finally, the advent of high density microarrays, advances in bioinformatics, and the discovery of molecular signatures for dysplasia and cancer have opened the door for inclusion and adaptation of these gene signatures into microfluidic lab-on-a-chip devices.

Recent independent studies carried out by various research groups including our laboratory indicate that OSCC cells have a unique gene transcription profile, which differs from that of normal cells.[30,33,34] These studies found a set of genes that are significantly down or upregulated in OSCC tumors.[30,33–35] To identify an OSCC tumor predictor, microarray data obtained from paired OSCC tumors and normal tissues was analyzed by the class prediction algorithm Support Vector Machines (SVM,[36]). SVM and the Goloub method were used to identify 25 genes with the highest predictive strength.[28,30] Cross-validation of patient tumor and normal data demonstrate that this

25-gene set predictor could classify tumor and normal samples with 96% accuracy. So far, microarray data from a total of 44 OSCC and normal specimens obtained from the University of Pennsylvania, the University of Pittsburgh, and data sets from Geo Data Set, NCBI have been tested. The 25-gene predictor correctly classified all samples producing average accuracy rates of 87% to 100%. Finally, using existing Affymetrix U133A chip derived data sets (NCBI Geo DataSets) from 20 other human tumors including breast, renal clear cell tumor, acute myeloid leukemia, lymphoblastic leukemia, and Barrett's associated adenocarcinomas resulted in accuracies of <25%. In short, by microarray analysis, a significant tissue-specific transcription profile from 25 genes that can accurately predict OSCC tumor and distinguish between OSCC tumor and normal tissue has been identified. This 25 gene predictor for oral cancer would be a good way to detect the presence of oral cancer in saliva using a lab-on-a-chip device. However, whether these same predictors can be used for detection of saliva derived pre-cancer and cancer cells has yet to be tested.

For human cancer, nucleic acid-based methods provide the most accurate means of detecting biomarkers. This is primarily due to the tremendous sensitivity and specificity of PCR and other nucleic acid hybridization-based amplification and assay techniques. Although in some cases, free-floating RNA can be detected in clinical samples, the utility of cell-free RNA as a diagnostic is controversial.[37,38] In general, the nucleic acids of interest are sequestered inside host cells. The processing and identification of these nucleic acids requires capture and isolation of target cancer cells; then lysis; nucleic acid isolation by, for example, solid phase extraction or hybridization; PCR; and amplicon labeling and detection.[39–46] Early work has indicated that mRNA from exfoliated saliva cells is somewhat degraded. This was expected since the cells assayed in this study were terminally differentiated oral epithelial cells.[47] Since pre-cancer and cancer cells present in saliva are more likely to be undifferentiated, they should still be actively manufacturing mRNA. In agreement with this notion, recent work by Spivack et al[48] has demonstrated that RNA can be isolated, amplified using reverse transcription-PCR and used for gene expression analysis from exfoliated cancer cells from the oral cavity. However, to date, no gene expression profiling using exfoliated cells obtained from saliva has been reported. Besides RNA, DNA from exfoliated cells has also been examined as a means to screen for oral cancers.

Regions of DNA instability, frequently found in many cancers, have been tested as a screen for oral cancer using saliva samples.[16,49] For example, using saliva obtained from tumor patients Okami et al[16] detected MSI in 80% of the samples. These results suggest that MSI has the potential to be used as a molecular screening for oral cancers. More recent work using a similar MSI approach has shown that loss of heterozygosity or MSI of at least 1 marker can be detected in 79% of the oral cancer saliva samples with no microstaellite alterations detected in normal saliva.[15] Thus, MSI appears to be a sensitive means of detecting cancer cells in the exfoliated cell population of saliva. Whether, MSI methods can be used to detect pre-cancer lesions has not been reported. Besides MSI, several other methods using DNA have been examined as potential screens for oral cancers.

DNA FISH has also been tested as an approach for screening oral cancers.[14] Recent work has shown that interphase FISH can detect chromosome imbalances associated with malignancy.[14] In addition to FISH, aberrant promoter hypermethylation patterns of cancer-related genes in saliva of head and cancer patients has been shown to be potentially useful in detecting and monitoring disease recurrence.[18] For example, Rosas et al[50] identified abnormal promoter hypermethylation in saliva samples from 65% of the patients with oral cancers. Finally, PCR was used to detect changes in mitochondrial DNA from exfoliated pre-cancer cells of the oral cavity. This worked demonstrated that mitochondrial DNA increases with histopathlogical grade. Thus, while PCR seems the most sensitive method for detection of pre-cancer and cancer cells in saliva, several DNA methods have potential applications for oral pre-cancer and cancer screening. However, more work is required to determine which of these DNA methods could be used in a lab-on-a-chip device.

MICROFLUIDICS AND LAB-ON-A-CHIP

Broadly, microfluidics technology—also referred to as lab-on-a-chip or micro-total-analysis systems (μTAS)—is the adaptation, miniaturization, integration, and automation of analytical laboratory procedures into a single device or "chip." Microfluidics is often regarded as the chemistry or biotechnology equivalent of the silicon integrated circuit chip that has revolutionized electronics, computers, and communications. Currently, microfluidic

systems are under development for disease diagnostics, controlled drug delivery, drug discovery, detection of bioterrorism agents, and air and water quality monitoring.[39,40,43–46,51–55] AQ3

In its most comprehensive medical use, the microfluidic diagnostic system would accept and process a small biopsy or a small sample of blood, saliva, lung aspirations, urine, or intraductal breast fluid; and then it would provide an easy-to-interpret readout indicating the presence and quantity of specific molecules, such as pathogen antigens, nucleic acids, antibodies, metabolites, toxins, drugs, and cancer markers, in the sample. The potential benefits of microfluidics derive from the use of small sample volumes, automated operation, short processing times, reduced reagent consumption, reproducibility and consistency, reduced exposure to hazardous materials or infectious agents, minimal risk of sample contamination, convenient disposal, operability by minimally-trained personnel, and low cost. Finally, microfluidics will bring the power of sophisticated analytical techniques to rural areas and developing countries where conventional analytical laboratories are lacking.

Some of the more fundamental engineering challenges and design issues for microfluidic systems are related to the propulsion, stirring, and flow control of the sample and reagents within the small (sub-millimeter) conduits of the chip. For example, within the microfluidic device, the stirring and mixing of reagents under the laminar flow conditions characteristic of microfluidics is a challenging problem. Microfluidic devices have incorporated various ingenious concepts, ranging from the miniaturization of macroscopic pumps and valves to novel propulsion techniques that utilize electro-kinetic, magnetic, surface (capillary), and osmotic forces.[56–59] Likewise, various microfluidic PCR thermal cyclers have been demonstrated, ranging from batch-mode, temperature-regulated chambers to continuous-flow reactors in which the PCR cocktail is pumped through a series of heated zones maintained at different fixed temperatures.[60,61] Another issue is the choice of detection modalities to be interfaced with the chip to identify the target molecules. Possibilities include laser-induced fluorescence, chemi- or bio-luminescence, electrochemical sensing, plasmon surface resonance, and piezo-electric transducers.

Simple "microfluidic" diagnostic devices such as lateral-flow and consecutive flow assays for home-pregnancy testing, drug abuse testing, prostate-specific antigen (PSA) detection, saliva-based HIV testing, as well as tests for bacterial contamination of food and water are already available on the market today.[62] The lateral flow strip comprises a porous nitrocellulose membrane or capillary network, striped with one or more capture zones of immobilized antibodies that specifically bind target molecules. Serum, urine, or saliva samples blotted onto the strip flow through the porous membrane by capillary action, and molecules contained in the sample bind to the capture zone. These molecules are tagged with secondary antibodies conjugated with labels such as carbon or gold particles, fluorescent/phosphors, or enzymes that convert a substrate compound to a luminescent reporter or electrochemical agent. These labels render the captured molecules visible to the naked eye or facilitate detection with an optical or electrochemical detector.

Although it is feasible to construct a completely self-contained microfluidic system that includes micropumps, microvalves, and detection devices fabricated directly into the cassette or chip, this approach is deemed uneconomical for medical diagnostics. Instead, an inexpensive, single-use, disposable credit card-sized microfluidic cassette is designed to mate with a portable instrument that provides fluidic power, flow control, regulated heating and cooling, signal detection, and data processing. Suitable microfluidic components are selected and integrated into the cassette according to: (1) the disease or ailment and the specific diagnostic information sought; (2) the biological basis of the clinical test (eg, immunoassay, cell sorting and detection, gene detection, and transcription or protein expression profiling); and (3) the sample type (eg, tissue, whole blood, serum, saliva, urine). By selection of appropriate PCR primers or antibodies and modifications of basic operating protocols, it is feasible to reconfigure the microfluidic system for diagnostics of many types of cancers based on protein or RNA detection.[63,64]

A fully-integrated lab-on-a-chip for cancer diagnostics will require integration of components for cell sorting (in order to deplete the sample of non-cancer cells and/or enrich the sample with the target cancer cells), cell lysis, nucleic acid isolation, multiplex RT-PCR or biobarcode amplification, and multiplex detection of labeled PCR products or sorted biobarcodes. For the most part, "on chip" processes to perform these assays have been described. For example, Anderson et al[41] have designed a credit card size chip that is capable of

extracting and concentrating nucleic acids from milliliter aqueous samples and performing microliter chemical amplification, serial enzymatic reactions, metering, mixing, and nucleic acid hybridization. Similarly, a microfluidic system that can perform cell lysis and DNA amplification was developed by Lee et al.[46] Furthermore, Liu et al have constructed a microfluidic cartridge that can automate the fluidic handling steps required to carry out a microarray gene expression study of human leukemia.[43] The fully integrated microfluidic device consists of microfluidic pumps/mixers, fluid channels, reagent chambers, and a DNA microarray silicon chip. Microarray hybridization and subsequent fluidic handling and reactions (including a number of washing and labeling steps) can be performed in this fully automated and miniature device before fluorescent image scanning of the microarray chip. In addition, Marcus et al have utilize microfluidics to isolate picogram and subpicogram mRNA templates, as well as to synthesize cDNA from individual cells while Chang et al have described a microfluidic chip that can perform PCR.[65,66] Finally, Tsai have developed a micro-RT-PCR (microRT-PCR) chip that is designed to quantitatively detect tumor viruses.[67] The next steps will be to integrate all these processes onto a single chip. To develop a cancer diagnostic lab-on-a-chip, for example based on transcriptional profiling of a sub-population of cancer cells in a clinical specimen, will more than likely require a time frame of 1 to 2 years to demonstrate feasibility, and another 2 to 5 years for the development of prototypes suitable for clinical trials.

LAB-ON-A-CHIP BASED ORAL CANCER DETECTION

Screening for and diagnosis of head and neck cancers using oral fluid samples provides an illustrative case study for developing a microfluidics cancer detection and analysis system. A device based on an inexpensive, disposable microfluidic cassette, such as the one shown in Figure 3, with pre-loaded, freeze-dried reagents, and used in conjunction with a handheld instrument would promote early screening and diagnostics during dental visits or routine medical exams. The patient would provide 1 mL of saliva, which is taken up by a sponge-tipped disposable collector. The collector is inserted into the cassette to inject the collected oral fluid through a sample inlet port. Within the microfluidic lab-on-a-chip device, antibodies conjugated to magnetic beads would

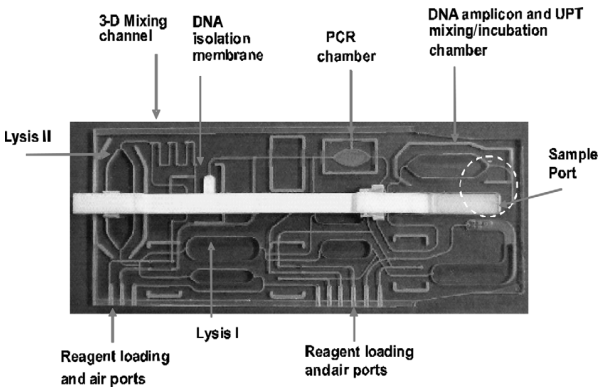

FIGURE 3. Example of a microfluidic lab-on-a-chip. The saliva sample is introduced into the sample port. The saliva sample is lysed with enzymes, detergent, and chaotropic salts in a two-step, two-chamber lysis process. The nucleic acids are isolated from the lysate by solid-phase extraction using a porous silica membrane as a nucleic acid binding phase. Purified nucleic acids eluted from the silica membrane are amplified by PCR using specific primers. The PCR amplicons are labeled with up-converting phosphor particles and conjugated to antigens, then run on a blotted nitrocellulose strip, where they are captured by immobilized antibodies and detected by a laser scanner. Similar pathogen detection chips, now widely developed by many groups throughout the world, can be adapted for cancer screening and serve as a basis for more sophisticated lab-on-a-chip cancer diagnostics systems. The chip pictured above is made of polycarbonate plastic and measures 10 cm in length and 3 cm in width. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

assist in removal of all lymphocytic cells. Next, the target pre-cancer and cancer cells would be captured and enriched from the sample using magnetic beads functionalized with antibodies to the specific dysplastic and cancer expressed cell surface glycolproteins (for example EpCAM). The cancer cells would be isolated and detected. The detection of cancer cells provides an initial indication of the presence of disease.

Subsequently, the isolated cancer cells would be lysed by thermal or osmotic shock, and their nucleic acid content isolated and purified using solid-phase extraction (eg, chaotrope-induced, selective binding of nucleic acids to a silica solid phase followed by elution of pure nucleic acids). Alternatively, the RNA released from the lysed cells could be hybridized to magnetic beads functionalized with poly-T to capture any mRNA or functionalized with complementary oligonucleotides to capture gene specific transcripts of interest. An external magnetic field will be used to manipulate the magnetic beads suspended inside the fluidic circuits of the microfluidic chip.

The isolated specific mRNAs are then be reverse transcribed and, when needed, amplified

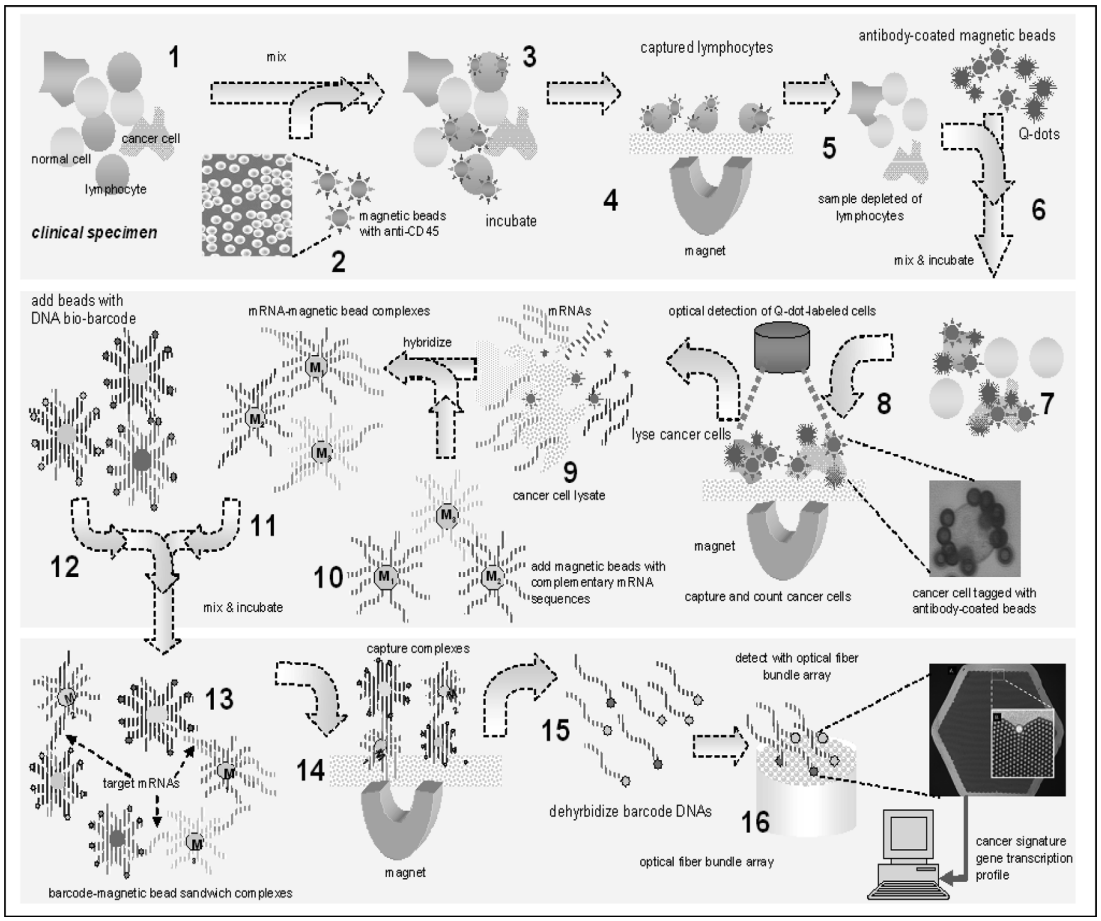

FIGURE 4. Cancer diagnostics format based on magnetic bead cell sorting and multiplex detection of a panel of mRNAs using bio-barcodes. The cell-laden sample containing normal cells, cancer cells, and lymphocytes is introduced into the microfluidic cassette [1]. The cell suspension is mixed [2] and incubated [3] with magnetic beads functionalized with anti-CD45 antibody that specifically binds to lymphocytes. An external magnetic field isolates the lymphocytes-magnetic bead complexes from the solution. [4] The supernatant, depleted of lymphocytes, [5] is mixed and incubated [6] with magnetic beads functionalized with antibodies to membrane glycoproteins specific to cancer cells (such as EpCAM and HSP47) and with quantum dots conjugated with the same antibodies as the magnetic beads or different antibodies specific to membrane proteins of cancer cells. The cancer cell-bead-quantum dot complexes are isolated from the solution with the aid of an external magnetic field. [8] The compartment is washed to remove any unbound quantum dots. An estimate of the number of cancer cells is obtained with a CCD camera. The immobilized cancer cells are lysed, [9] and then mixed and incubated with magnetic beads functionalized with oligonucleotides complementary to a pre-selected set of mRNAs and to house-keeping genes. [10] The magnetic beads with captured mRNAs are isolated by application of an external magnetic field and thoroughly washed. [11] The captured mRNAs are hybridized with nanoparticles with oligonucleotides complementary to the specific cancer cell mRNA and the barcode DNAs [12] to form barcode-magnetic bead-selected mRNA sandwiches. [13] The magnetic beads are immobilized and the solution is washed. [14] The barcode DNAs are removed from the beads [15] and detected by fluorescence using a fiber optic array. [16] The measured profile is compared with archived gene transcription profiles to determine the cancer type and stage. The detection method described here combines the bio-barcode format of Mirkin and coworkers[64] and the fiber optic bead array of Walt and coworkers.[68] Figure drawn by M. Mauk, 2006. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

with linear RNA amplification. The expression levels are detected with multiplex Real Time-PCR or with non-enzymatic techniques such as bio-barcodes. Figure 4 depicts a schematic of a bio-barcode, bead-based process for isolation of total mRNA, followed by multiplex amplification and detection of a panel of mRNAs. Multiplexed, non-enzymatic amplification of DNA with bio-barcodes is a less demanding assay than multiplexed PCR.[63,64]

Multiplexed DNA (or protein) detection with bio-barcodes will provide a gene transcription profile that is highly predictive of oral cancer. Amplification products can be labeled and detected by fluorescence, phosphorescence, chemi- or bioluminescence, or electrochemical sensing. For

example, the amplified gene transcripts can be detected and quantified by a hybridization-based optical biosensor such as miniaturized fiber optic bundle arrays.[68] These fiber optic sensors serve as a low-cost alternative to gene chip microarrays and can be efficiently interfaced with and optically coupled to a microfluidic chip.

The transcription profile of the isolated sample cancer cells will be compared with cancer signature profiles archived in a database using established statistical rules to identify the type of cancer and to discriminate between cancer and normal samples. This method will provide near-real time diagnostic reports in the form of specific gene transcription signature data in 10 to 60 minutes.

It is also important to note that the microfluidic devices described here could be easily adapted and tailored for the diagnosis and analysis other cancers and monitoring treatment.

FUTURE APPLICATIONS AND DIRECTIONS

In the long term, we envision a detection system that consists of an inexpensive, single-use, disposable cassette that operates in conjunction with a moderately priced, portable analyzer. Various types of cassettes will accommodate different tests to be identified through a bar code on the cassette. The bar code will direct the analyzer to carry out an appropriate sequence of operations. By keeping the analyzer generic, it would be possible to reduce its cost; approximately \$50 for each test cassette and a one time cost of \$5 to \$2000 for the analyzer.

In addition to applications for cancer detection including point-of-care screening, the lab-on-a-chip will provide a means for cancer typing and staging, monitoring of disease progression and recurrence, assessing the effectiveness of therapies, and detecting drug resistance. In the operating room, the chip will aid the surgeon in defining the clear boundaries after tumor extraction and assist in the assessing whether the primary tumor has spread to the lymph nodes. Finally, with the incorporation of the appropriate assays and detection systems, the lab-on-a-chip could be used to detect gene mutations, hetero- or homozygosity, loss of function, chromosomal translocations, cancer-related methylation, and other alterations that have been shown to play a role in oral cancer development and progression.

Research, development, and optimization of microfluidic diagnostics technology continues. Workable, albeit perhaps not optimal, components for the lab-on-a-chip operations necessary to process clinical samples for oral cancer diagnosis, such as magnetic-bead based cell sorting, protein and nucleic acid isolation with magnetic beads and/or solid-phase binding media, incubation and temperature cycling, reverse transcription, quantitative PCR, and enzymatic and non-enzymatic signal amplification and detection, have been adequately demonstrated. The remaining major challenge is to seamlessly integrate the various components into a single microfluidic cassette amenable to automated operation and optimize multiplexing capability for 5 to 30 protein or nucleic acid targets.

Microfluidics appears poised to make a significant impact on the technology and practice of oral cancer screening and diagnostics and on heath care in general. In fact, a few simple microfluidic diagnostic devices are now available, or are reaching demonstration stages in academic and industrial laboratories. Unfortunately, there are still many questions regarding the identification of various expression profiles, establishing their predictive power, and developing procedures to collect, process, and analyze specific cancer samples and derive clinically useful information utilizing these cancer markers. Synergism from coordinated development of practical lab-on-a-chip systems in parallel and close collaboration with supporting and exploratory biomedical and clinical research would foster progress in both microfluidics technology and cancer diagnostics and therapeutics. The advent of low-cost, mass-produced, and convenient-to-use microfluidic cancer diagnostic systems would accelerate cancer marker research and facilitate broad-based clinical trials with large numbers of human subjects which ultimately would lead to decreases in patient morbidity and mortality.

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