A novel platform for nucleic acid biomarker-based diagnosis of thyroid cancer

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Abstract

The standard of care for patients with clinically significant thyroid nodules is an ultrasound-guided fine-needle aspiration biopsy with cytological evaluation. Nearly 20% of the patients are found to have indeterminate cytology requiring surgical thyroidectomy to exclude malignancy; nucleic acid-based biomarkers to improve diagnosis are promising, but there is clearly a need for improved technology to bring these into common clinical practice. Here, we demonstrate the use of a thermoplastic disposable microfluidic chip to extract nucleic acids from thyroid cancer cell lines and thyroid tissue for potential use as a rapid cancer diagnostic method. The microsolid phase extraction chip was used to extract microRNA, mRNA and DNA from the well-characterized thyroid cancer cell line BCPAP and from human thyroid tissue obtained during surgery. In our study, we successfully used the microchip to extract and detect thyroid cancer-associated nucleic acids from as few as 50 to 500 cells (fewer cells than in a typical 1-ml fine-needle aspirate) and from human thyroid specimens. Our data suggests that this micro fluidic platform may serve as a technology that may improve the speed, reliability and cost of thyroid cancer diagnosis in cases of indeterminate cell aspirates.

Introduction

Cancer is a leading cause of morbidity and mortality, with an estimated 1.6 million new diagnoses and 577,190 deaths in the United States alone in 2012. Thyroid cancer is the most common endocrine malignancy; the number of new cases and deaths from thyroid cancer in the United States in 2012 were estimated to be 56,400 and 1,7401–3. The majority of patients with thyroid cancer have papillary thyroid cancer (PTC)4,5. Early diagnosis of cancer has been shown to improve disease outcome and is a key resource for physicians in the treatment of cancer6. The current gold standard of detection of thyroid cancer is fine-needle aspiration biopsy (FNAB) to retrieve cells from thyroid nodules, followed by cell fixation and microscopic examination of cell morphology by a trained cytopathologist. This process is initiated after a physician’s physical examination and record of the patient’s case history raises suspicion of potential thyroid malignancy; this is often supplemented with other laboratory tests including serum thyrotropin levels for thyroid function and/or thyroid evaluation using ultrasound4. However, the determination of thyroid cancer in an individual is particularly problematic since the gold standard method of FNAB cytology has a relatively high rate of indeterminate results, ranging from 10% to 25%. Poor FNAB specimens, those with indeterminate cell morphology, unusable slide smears or insufficient cell clusters, which present a non-diagnostic result, lead to surgical resection to rule out malignancy. Only approximately 8%–20% of these patients undergoing surgery are diagnosed with cancer5,6. This dictates the need for a less invasive, non-surgical procedure to improve the diagnostic use of the FNAB specimen.

Nucleic acid cancer biomarkers have risen to the forefront of approaches to improve cancer diagnostics, particularly in the area of point-of-care (POC) cancer diagnostics. The vast array of data from transcriptomic network studies in cancer cells versus normal cells has led to the discovery of biomarkers of various biomolecular species including protein, mRNA, micro RNA (miRNA)7 and DNA8–11. The availability of validated biomarkers has generated the use of these molecules as biosensors for detecting upregulated or down regulated levels of cancer-associated proteins such as prostate-specific antigen in prostate cancer; DNA mutations such as BRCA1 and BRCA2 in breast cancer11 and miRNA12. In addition, cell-free nucleic acids present at high concentration in the blood of cancer patients have been associated with tumour progression and are being investigated for clinical utility in cancer diagnosis and prognostication. While no single biomarker or class of molecules has been discovered with 100% predictive accuracy for cancer diagnosis, the use of a panel of multiple markers comprising protein, mRNA, miRNA and DNA in some combination increases the predictive value and decreases false-positive and false-negative diagnoses6,11,13–19.

Molecular diagnosis has emerged as a potentially useful tool to evaluate cytologically indeterminate thyroid nodules20. Two novel genetic methods to evaluate indeterminate thyroid nodules have recently become commercially available21,22. These methods are based on molecular tests using
material from FNABs. However, both are expensive and require send-out to a central laboratory and several weeks for processing. There is therefore an urgent need for a POC biomolecular diagnostic platform that satisfies the criteria listed by the World Health Organization: simple-to-use, rapid, low-cost, sensitive, accurate, specific and robust. Most current methods for using low-volume human surgical specimens to diagnose cancer are limited to low-throughput configurations or require expensive, send-out tests that take weeks to return.

In this study, we highlight a novel technology that can achieve the following: 1) potentially decrease the incidence of inconclusive FNAB diagnoses in thyroid cancer by requiring very few cells (5–500) for analysis and improving the diagnostic capacity above cytological evaluation alone; 2) enable detection of three different classes of nucleic acid biomarkers from these low-volume samples and 3) be adapted as a future POC system for these low-volume samples. We employed nucleic acid biomarkers of diagnosis at the patient's bedside.

We demonstrated our ability to detect these biomarkers with high sensitivity using our platform. The platform is a micro-solidphase extraction (µSPE) column housed in a thermoplastic disposable chip adapted as a future POC system for a central laboratory and several weeks to return.

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performed using 1-ml plastic syringes (BD, Franklin Lakes, NJ, USA) and a syringe pump (PHD Ultra; Harvard Apparatus, Holliston, MA, USA) at a flow rate of 0.8 ml/h unless otherwise noted.

Before introducing the sample, the μSPE column was buffered with 150 μl of 1.5 M guanidine thiocyanate (GuSCN), 50% 2-propanol and 1× RNA secure (Applied Biosystems, Foster City, CA, USA). Next, the 100 μl cell sample was mixed with 300 μl of lysis buffer for a final concentration of 1.5 M GuSCN, 50% 2-propanol, 1× RNA secure and 6 μg carrier RNA (Qiagen, Valencia, CA, USA). The cell-lysis buffer mix was loaded through the column to mechanically and chemically lyse the cells and bind miRNA and mRNA to silica particles within the column. This was followed by sequential washes with 50 μl of 70% ethanol and 50 μl of 100% ethanol. The column was air dried for 30 min by pushing air through an empty syringe at a rate of 1 ml/h. Finally, two 30 μl elution washes with 50 μl of 70% ethanol were performed directly in LB broth. The lysis buffer was changed to 0.5 ml/h. Data are presented only for the first fraction as both fractions yielded similar results.

Quantitative detection of isolated nucleic acids by qPCR

The extracted nucleic acids were assayed off-chip. For mRNA, all reagents and cycling conditions for quantitative reverse transcription-polymerase chain reactions (qRT-PCRs) were used as recommended in the protocol for TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA). We chose miR-222, a miRNA known to be upregulated in both thyroid34,35 and breast cancer36, and the endogenous control miRNA RNU44 as our targets to demonstrate miRNA isolation from cancer cell lines on-chip using two-step qRT-PCR. Cells were extracted in 3 μSPE channels each, ranging from 5 to 500,000 cells for both cell types. Extracted miRNA was reverse transcribed with the TaqMan MicroRNA Reverse Transcription Kit with 5 μl of the RNA eluate. RT primers (5×) were specific for RNU44 (part no. 4427975, assay ID 001094) and miR-222 (part no. 4427975, assay ID 002276). The resulting cDNA (1.33 μl) was amplified using the TaqMan Universal PCR Master Mix II (4304437; Applied Biosystems) using 20× primer and probe sets for RNU44 or miR-222 from the assay IDs listed above. The positive control reaction included in each RT-PCR contained QPCR Human Reference Total RNA (750500; Agilent, Santa Clara, CA, USA) as the template. Negative controls included the following: 1) reactions lacking only the RT enzyme but including the positive control template and 2) a no-template control including all reagents but water instead of a RNA template. The routine method of 2^−ΔCT was used to analyse test miRNA expression relative to an internal control, RNU4437. The (−) ΔCT values were graphed to indicate normalized expression levels of the test miRNA.

For mRNA, extracts were assayed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA after DNase treatment (TURBO DNA-free kit; Ambion, Inc., Austin, TX, USA) to reduce amplification of contaminating DNA during PCR. First, 10 μl of the RNA eluate was DNase treated in a 15-μl reaction volume of which 5 μl was used in triplicate one-step qRT-PCR reactions. The 2× Brilliant II qRT-PCR Master Mix (600804; Agilent) was used with 20× primer and probe set for GAPDH (Hs 99999905_m1; Applied Biosystems). One-step qRT-PCR reagent and cycling conditions were followed as described by the manufacturer: qPCR Human Reference Total RNA was diluted and used to generate a standard curve for quantification of mass of RNA from cycle threshold (Ct) of amplification. Negative controls were no-template water reactions and no-RT enzyme reactions.

Extracted DNA was detected using RNase P reference gene (4316831; Applied Biosystems). TaqMan Universal PCR Master Mix II was used as described in the manufacturer’s protocols with 20× RNase P primer and probe. Control human male DNA (4312660; Applied Biosystems) was diluted, added as the positive control template and used to generate a standard curve for quantitation of DNA mass in extractions. Negative controls were no-template controls including all reagents but water instead of a DNA template. All qRT-PCR and quantitative real-time PCR (qPCR) reactions were performed in an ABI 7300 real time PCR instrument (Applied Biosystems).

BRAF DNA assay

The BRAF DNA mutation T1799A was detected by allele-specific-end point PCR (AS–PCR) as previously described38,39. The genetic assay detects the mutant sequence if ≥1% of the mix of mutant and wild-type DNA contains the mutation and it is done in duplicates: separate PCRs for the forward and reverse strand. As purity and DNA yield may be adversely affected by blood and other sample inhibitors, the quality of blanked specimens or non-optimal DNA extraction protocols,

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GAPDH is primed in the same reaction to determine the quality of the DNA preparation as an internal positive control. A positive sample will yield both products for GAPDH (247 bp) and the mutant BRAF allele at 198 bp (forward strand) or 152 bp (reverse strand) in the same reaction. Samples were amplified at 4 different input volumes in the PCR mix (1, 2, 6 and 10 µl of eluted DNA per 25 µl reaction volume) for the reverse strand to avoid non-amplification from inhibitors or from too few copy numbers of the target loci. Samples positive for reverse strand AS–PCR were tested for the forward strand PCR. Each PCR contained 1× AmpliTaq Gold 360 Master Mix, 10 pmol each of BRAF forward and reverse primers and variable amount of DNA template and water for a 25-µl volume reaction. Reactions were cycled in a thermocycler at 95°C for 10 min, followed by 45 cycles at 94°C for 20 s, 62°C for 30 s, 72°C for 25 s and extension at 72°C for 5 min.

**Human thyroid specimen characterization**

Patient specimens were obtained under the Boston University Institutional Review Board approval, following patient consent. Thyroid tissue from papillary thyroid carcinoma and benign thyroid nodules was obtained from 24 patients undergoing surgical resection during the course of routine clinical care. The samples were snap-frozen in liquid nitrogen and stored at −80°C. For nucleic acid extraction from thyroid human specimens, 10 mg of patient tissue was quick-thawed at 37°C, homogenized with an 18-gauge blunt needle and syringe and mixed with lysis buffer as detailed below. Extraction of nucleic acids then occurred on-chip as detailed above. For miRNA, we selected specimens from 7 patients known to have PTC and 7 patients known to have benign thyroid nodules for analysis. We assayed for 4 miRNA (miR-222, miR-221, miR-146b and miR-375) using the miRNA technique as described above. For DNA, we selected specimens from 13 patients known to have PTC and 4 patients known to have benign thyroid nodules for analysis. We assayed for 2 loci, GAPDH and mutant BRAF, in a multiplex AS–PCR as described above. For the microfluidic extraction, positive and negative specimens were tested in a random order and each specimen was tested on a new chip to avoid cross contamination. The samples selected for testing were not chosen with regard to any parameter other than their histological diagnosis.

**Results**

The goal of on-chip extraction is to maximize the amount nucleic acid extracted from as few cells as possible, while maintaining the integrity of the nucleic acids extracted. Our goal here was to determine the analytical limit of detection for the single-use microfluidic chip for extraction of nucleic acids. Before working with the human specimens, the assay was optimized using cultured human thyroid and breast cancer cell lines.

**miRNA is detectable in as few as 50 cells from cultured human cancer cell lines**

RNU44 miRNA, a stably expressed internal control, was detectable in as few as 50 cells in breast cancer (MCF-7) and thyroid cancer (BCPAP) cells (Figure 1a). In comparison, miR-222 was detectable in as few as 5000 breast cancer (MCF-7) and 50 thyroid (BCPAP) cancer cells. As expected, miRNA extraction was similar across all thyroid cancer cell densities as
Nucleic acids are detectable using our chip in human thyroid cancer specimens

After determining the cell number detection limits for miRNA, mRNA and DNA, the chips were tested for miRNA and DNA isolation from patient thyroid specimens. A total of 14 patient samples (7 benign thyroid specimens and 7 PTC specimens) were selected for testing miRNA extraction with the microfluidic assay. All tissue extracts had measurable levels of miRNA (Figure 3) and all of the 4 miRNA selected were found to have expression at some level in both the malignant and benign thyroid specimens. The levels of these miRNA were variable in the 7 malignant tissues (Figure 3a). However, 6 out of 7 malignant thyroid specimens had upregulated miR-222; 4 of these also had upregulated miR-146b expression. Three of these miRNA were found at higher levels in normal/benign tissues than the malignant tissues (miR-222, miR-146b and miR-375); one of the miRNA was consistently found at lower levels in normal/benign thyroid nodules than malignant specimens (miR-222). However, these observations were not found to be statistically significant.

We tested 17 human thyroid specimens for human genomic DNA by GAPDH–PCR; all were found to be positive (Figure 4c). The BRAF mutant sequence encoding V600E was found in 23% (3/13) of the malignant tissues by both forward (Figure 4b) and reverse (Figure 4a) strand AS–PCR, which correlated with their official histopathological findings of BRAF positivity. The 10 malignant human thyroid cancers found to be negative for the BRAF mutation yielded only the top band for GAPDH at 247 bp and had detectable Ct values for RNase P; this also correlated with mRNA yields. The limit of detection for extracted DNA was lower for MCF-7 at 50 cells than BCPAP at 500 cells.

**Figure 2:** Detection and quantification of mRNA and DNA from MCF-7 and BCPAP cells. The average Ct from qPCR amplification is shown and the total amount of nucleic acid (pg) in (a) 30 µl of mRNA and (b) 50 µl of DNA eluate is plotted for each cell density (5–500,000 total cells) in triplicate chip extractions. The qPCR targets were housekeeping GAPDH mRNA and RNase P DNA.

Figure 3: Expression of 4 selected miRNA in thyroid nodule tissue extracted on-chip from triplicate qPCR validations for 14 individually analysed patient samples (differentially coloured). miR-222, miR-221, miR-146b and miR-375 levels (solid and patterned bars) relative to RNU44 endogenous control are shown for (a) 7 malignant human thyroid specimens and (b) 7 normal or benign human thyroid specimens.

their official histopathological findings of BRAF negativity. The DNA yield (pg) from the extracted tissues had a wide distribution with an average of approximately 1100pg of DNA (200–2040 pg, 95% CI) collected in the first 50 µl eluted (Figure 4c). Diluting the DNA 2- to 4-fold in the AS–PCR reaction did not result in improved end-point amplification; however, increasing the DNA concentration 3- to 5-fold led to stronger amplification for certain samples (data not shown). This suggests that the eluted samples had minimal inhibitory substances such as haem and its derivatives from blood.

Discussion
The microfluidic chip presented here is the first crucial step towards a complete micrototal analysis system to allow for POC diagnosis of thyroid cancer. Such a system would afford the patient a better tolerated and non-operative procedure, quicker diagnosis with less time in and out of the clinic, more accurate and conclusive biopsy results and lower costs in terms of materials, equipment and manpower. The µSPE chip requires less biological sample compared with most bench-top sample preparation methods (10 mg tissue or <1000 cells vs. 100 mg tissue and >100000 cells) for reliable nucleic acid extraction. The cost of the chip material is insignificant compared with the cost of the consumables and instrument required for the traditional commercial kits. In addition, because of the sample preparation chip’s simple design, a microchannel with pressure-driven fluidic control, it may be easily integrated into a complete POC system.

Much of the focus for cancer POC biosensor development has been on protein analytes due to the existence of a more extensive set of validated protein tumour markers compared with other classes of biomolecules. However, the clinical utility of many potential markers across different classes continues to be evaluated for their sensitivity and specificity to various cancers. In total, a panel of DNA, miRNA, mRNA and protein tumour markers maybe envisioned in future as providing not only diagnostic utility but also information regarding tumour staging, disease recurrence and treatment choices or personalized medicine. Therefore, the results of this study are consistent with a simply designed, easy-to-use, disposable, cheap and rapid method that incorporates the capability to provide information on multiple classes of biomarkers.

A key advantage for thyroid cancer diagnosis in our system are the low limits of detection achievable, ranging from 5 to 500 tissue culture cells and approximately 10 mg of tissue, for detection of miRNA, mRNA and DNA. These limits are well below the usual number of cells provided in most FNAB and fixed or frozen tissue specimens. Beyond enabling the detection of miRNA, mRNA and DNA, the µSPE chip extracted material was of sufficient purity for qPCR. This was true for both tissue culture cells...

Figure 4: DNA biomarker detection in thyroid tissue extracts. DNA was extracted on-chip from individual patients and eluted in two 50-µl fractions per patient. Fractions were assayed for mutant BRAF by allele-specific (AS)–PCR using oligonucleotides complementary to the (a) reverse strand and the (b) forward strand sequences encoding the V600E mutation. Representative 3% agarose gels are shown for both fractions from a subset of patients. Patients who were positive for the BRAF mutation had a 152-bp product in reverse strand AS–PCR and a 198-bp product in forward strand AS–PCR. GAPDH DNA was simultaneously amplified (247bp) in the same PCR reaction for each extract and served as an internal positive control for human DNA in the extract. For all PCR assays, the positive control DNA, (+)C, was from a known human thyroid cancer sample. Two negative controls were included in each PCR as shown in (a). These were a no-template control (−)1 and non-specific genomic DNA (−)2. (c) The amount of DNA present in the first 50 µl elution was determined by qPCR of the RNase P gene; the mean pg of DNA and 95% CI from 17 individual patient sample extracts is shown.

(miRNA, mRNA, DNA) and thyroid tissue (miRNA, DNA) and allowed quantification of the target in each assay. As the variable expression levels of these biomarkers represent the molecular profile of the patient's tumour (benign vs. malignant, prognosis to certain drug therapies, etc.), quantitative information is highly beneficial. To demonstrate the potential for clinical utility of our approach, we chose biomarkers with high specificity for PTC, including miR-221, miR-222 and miR-146b, and the BRAF T1799A (a transversion mutation resulting in the V600E codon mutation) in 26,41. The use of optimized qPCR probes to detect nucleic acid targets for miRNA and mRNA also increased the sensitivity of this PCR-based approach and the use of AS–PCR for the BRAF DNA assay is a common method currently in use at clinical laboratories with equivalent or greater sensitivity than Sanger sequencing and pyrosequencing42.

In this study, expression of the biomarkers was highly variable, even among the histological subtypes. Although the BRAF V600E mutation is highly specific and has become accepted as a PTC marker, it must be noted that its accuracy may not be high (approximately 45%); it ranges from 30% to 80% depending on the population41,43. We have observed similar discrepancies in the literature regarding the variability of miRNA and mRNA expression, even in published biomarker panels. Interestingly, differences exist between miRNA upregulation in PTC tumours; upregulated miR-146b was detected in tumours with mutant BRAF, although miR-221 or miR-222 may not be detected25,26,44,45. Unlike the BRAF V600E mutation and other DNA alterations in RAS, RET/PTC and PAX8/PPARγ, which are listed by the American Thyroid Association as molecular markers that may be considered in the management of patients with indeterminate FNAB cytology, the diagnostic utility of miRNA, in general, is promising but not yet fully adopted46. Therefore, our findings using small-volume specimens correlate well with the published results.

The development of a full POC thyroid cancer test will require simultaneous optimization of chip design, development of protein detection and a continued effort in the medical and biomarker discovery communities to identify additional specific and sensitive tumour markers such as those identified from micro array and protein array analyses. POC testing offers the advantages of faster diagnosis, limited reagent use, higher throughput, portability, ease of use and disposability. Our future work will add to the detection of proteins on-chip for integration with the current nucleic acid sample preparation chip and to create a chip where the entire process from specimen preparation


44. Chou CK, Chen RF, Chou FF, Chang HW, Chen YJ, Lee YP, et al. miR-146b is highly expressed in adult papillary thyroid carcinomas with high risk features including extrathyroidal invasion and the BRAF (V600E) mutation. Thyroid. 2010 May;20(5):489–94.