J18138; IRB00172441; NCT03863522
Automated Method for Breast Cancer Detection

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Version:	November 7, 2018

TABLE OF CONTENTS

1. Background, Hypothesis, Rationale and Objectives

1.1 Background

1.1.1. The Problem

In underdeveloped and developing countries of the world, breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death. in 2008, 691,000 new cases were detected and 269, 000 women succumbed to the disease [1]. Breast cancer incidence is rising in underdeveloped world because of longer life expectancies, decreased burden of infectious diseases, and changes in reproductive risk factors [2, 3]. patients with breast cancer in the underdeveloped world experience longer diagnostic delays than patients in developed countries, leading to later-stage presentations [4]. However, optimal early detection strategies are not well-characterized in settings where population-based mammography screening is not yet available, primary care services are limited, and pathology and treatment services are available only at regional hospitals [5]. In many of these settings, health care systems to address breast problems and efficiently refer patients with symptoms concerning for cancer are in their infancy [6]. currently, there are delays for up to 10 months in certain areas due to the lack of easy access to a clinical pathology laboratory or a pathologist [1, 7].

In remote areas with highly overworked pathologists, a new, inexpensive, accurate and rapid test for use in screening clinics to detect malignancies would greatly assist in prioritizing those patients for quick and detailed evaluation at the regional hospital [8, 9]. Currently, there are no tests for accurate and quick determination of an abnormal lesion in the breast as benign or malignant in the underdeveloped world. We have shown that DNA methylation that occurs specifically in breast cancer can serve a powerful marker for early detection of breast cancer in body fluids such as nipple aspiration, ductal lavage and core biopsy.

1.1.2 DNA methylation

DNA methylation is a molecular modification of DNA that is tightly associated with loss of gene expression [10]. The Sukumar lab has performed extensive work to derive methylated gene marker panels [11-17]which are specific to invasive ductal and lobular cancers. However, little work has been spent studying the methylation status of mammographically suspicious, biopsy proven benign lesions, and to select sensitive and specific markers that distinguish between malignant and benign lesions. Euhus and group have performed the most extensive analysis [18, 19] of this question. In one report [20] they studied DNA methylation of cyclin D2, APC, HIN1, RASSF1A,

and RAR-β in 290 benign and malignant breast epithelial cell samples obtained from palpable lesions by fine-needle aspiration (FNA) biopsy from 164 women. They concluded that tumor suppressor gene methylation increases in benign breast epithelium of high risk women. But could not identify any marker that clearly distinguished between benign and malignant lesions [20]. Markers that accurately distinguish between benign and malignant breast cancer are not available, and needed to be developed.

1.1.3 DNA Methylation Marker Selection and a method for detecting methylated genes

Towards the goal of developing markers that are specific to malignant breast cancer, Sukumar and co-workers identified methylation markers that are frequently methylated in breast cancer by performing large-scale methylation microarrays containing probes for 27K and 450K methylated regions (Illumina) in the genome. They also developed a sensitive and specific Quantitative Multiplex Methylation-Specific PCR (QM-MSP) technique [11, 15-17]. This method involves sodium bisulfite treatment of DNA, followed by a two-step reaction: multiplex PCR that amplifies both the methylated (M) and unmethylated (U) alleles of up to 14 genes in one reaction, followed by Q-PCR using primers and dual fluorophore labeled probes that amplify both the M and U alleles for each gene in a single well (22). The major advantages of this technique are: a wide dynamic range, its ability to detect a few M alleles in the midst of a vast excess of U alleles, and feasibility in small epithelial samples such as NAF, core biopsy and rFNA.

1.2 Hypothesis

Many underdeveloped areas of the world lack both the equipment and trained personnel required by standard diagnostic methods like mammography. We hypothesized that well-chosen methylation markers, incorporated into an inexpensive, automated molecular test that could be applied in conjunction with ultrasound at the point of care, even in low resource areas could achieve sufficient accuracy to prioritize patients with suspected malignancy for quick and detailed evaluation at the regional hospital.

1.3 Rationale and Preliminary Data

Assaying hypermethylated genes in fine needle aspirates of suspicious breast lesions that have been detected by ultrasound could help rapidly identify malignant tumors, where the methylation marker test is predicted to be positive. On the other hand, the test will score negative for the methylation markers if lesion is benign since the lesion would contain low or no detectable levels of methylation (9),(6;7). In the last two years, using QM-MSP, we analyzed training and test sets of primary tumors from USA, China and Africa and developed an optimal methylated gene marker panel whose performance is at, or exceeding 90% sensitivity and specificity.

1.3.1 The Cepheid cartridge

CEPHEID is a diagnostics company in California that has developed cartridges for the molecular detection of a large number of infectious diseases bacteria and viruses, including Anthrax and Ebola, with FDA approval for a large number of them. The cartridge is able to perform sample extraction, and PCR-based detection of the desired analyte- RNA, DNA or cDNA. The dye detection limit is less than 1 nM for most analytes.

The GeneXpert® System is available in a one, two, four, or 16-module configuration. All use the same GeneXpert analysis module, and the same patented cartridge technology. The GeneXpert® System returns most test results in about an hour, including sample preparation. The systems deliver results even faster than many alternative technologies, such as EIA or Immunoassay. The cartridges are single use, and detect up to 6 genes in the same cartridge through use of multiple calibrated fluorphor dyes with distinct excitation ranges. Thus, we will use them for detection of multiple methylated genes from the same section of the core biopsy or FNA collection from the breast.

Figure 1:
Left: GeneXpert cartridge.
Right: A two cartridge module- GX11-2-L of the GeneExpert Diagnostic System with laptop is shown. An 8- and 16-module machine is currently installed in the Sukumar Lab.

1.3.2 Optimization of the Cepheid cartridge for breast cancer detection

Next we optimized the conditions for analysis to achieve the lower limits of detection of methylated genes in the CEPHEID GeneXpert® cartridge. Further, we completed analysis of fine needle aspirates and touch preparations of xenografts of human breast cancer cells and of fine needle aspirates performed in surgically resected primary breast cancers very successfully in the cartridge. The cartridge is loaded with sodium bisulfite treated DNA, and performs the steps of QM-MSP within 3 hours, in contrast to the one week’s meticulous work by an expert technician.

1.3.3 Determination of receptor status

In addition to determining the methylation status of the tumor, sections or tissue lysate of the same lesion can be used in a separate cartridge on the GeneXpert® subtyping device to determine estrogen receptor (ER), progesterone receptor (PR), and HER2 status of the tissue which are key pathologic features responsible for determining treatment options. Thus, the assay can be successfully employed in underserved areas of the world in the future. This assay is completed in 1.5 hr. We tested FNAs of xenografts and successfully determined the status of the four markers in this cartridge for several breast cancer cell lines.

Summary: Identifying the optimal set of 10 methylated gene markers suitable for use in the US, China and Africa has been completed. Preliminary analysis of genes in the cartridge to determine their performance on FNAs was successful. With our help, CEPHEID has designed two cartridges to analyze a total of 10 genes by a quantitative multiplexed methylation specific PCR that uses actin as the internal housekeeping gene control. Preliminary analysis of FNA in the tumor subtyping has been partially successful. While the assay works with high level of accuracy using FFPE sections, conditions for optimal detection of the subtyping markers in FNA needs further work.

1.4 Objectives

1.4.1 Primary objectives:

1) To determine if DNA methylation profile of 10 genes in the automated GeneXpert Cancer Detection cartridge correlates with diagnosis based on the gold standard of histopathology of the core biopsy or resected sample.
2) To determine if DNA methylation profile of 10 genes as determined in the automated GeneXpert Cancer Detection cartridge correlates with FNA cytology.

1.4.2 Secondary objectives:

1) To compare the results of gene methylation based prediction of the FNAs as malignant or benign by the GeneXpert Cancer Detection cartridge to our known laboratory assay, quantitative multiplex-methylation specific PCR (QM-MSP).
2) To compare QM-MSP results of FNA with QM-MSP results of section of the biopsy or resected tissue.
3) To determine if the expression of ER/PR/Her2 and Ki67 in FNAs of suspicious, methylation positive breast lesions correlates with expression of ER/PR/Her2 and Ki67 in FFPE sections of tumors.

2. Selection of Patients

2.1 Eligibility Criteria

For this study, we require a total of 165 cases (women with invasive cancer) and 165 controls (women with benign breast disease), 18 years or older who have been recommended for ultrasound guided core needle biopsy of a suspicious breast lesion. Based on estimates that 40-50% of suspicious breast lesions will be malignant, we expect to enroll between 382 and 447 total subjects to provide the necessary cases and controls. Accordingly, after enrolling 382 subjects we will calculate rates of malignant and benign disease, and estimate total enrollment requirements, and continue enrollment as needed. See statistical considerations for additional details.

2.2 Ineligibility Criteria

Women may be excluded for any condition that in the opinion of the investigator may not make it safe to take part (e.g. comorbidity where stopping a concomitant medication is not in the best interest of the patient).

2.3 Subject Recruitment

Subjects will be recruited through the Johns Hopkins Imaging Center site at Green Spring. Women will be offered participation if they are recommended to have a breast core needle biopsy of a suspicious lesion.

3. Patient Registration

- Patients will undergo an informed consent process and subject registration number will be assigned.
- Registered patients will be tracked by patient log. This log will be submitted to the CRO on a periodic basis (quarterly, at minimum) until study closure and will include:
  - Subject name (or initials) ○ Registration number ○ Date of registration
  - Date of birth
  - Race

4. Study Calendar

	Baseline	Day of Procedure	Follow-Up 1[(1)]	Follow-Up 2[(2)]
Informed Consent	X			
Eligibility Confirmed	X			
Patient registration	X			
PROCEDURES:				
- Fine Needle Aspiration (FNA)		X		
- Core needle biopsy of suspicious lesion		X		
OTHER ASSESSMENTS:				
Adverse Events call			X	X

[1]: 1-2 days after procedure
[2]: 10-14 days after procedure

5. Study Parameters

5.1 FNA Procedure: For Suspicious lesions

Women ages 18 or older who have been recommended for ultrasound guided core biopsy for a suspicious breast lesion at the Green Spring Johns Hopkins Imaging site will be recruited into the study.

The FNA will be performed on one ultrasound visible lesion per patient, using the method described by Fabian et. al. [21, 22] which is standard at Johns Hopkins and used the by the breast imagers. When recommended for the core biopsy, participants are asked to abstain from aspirin and other medications/supplements related to blood clotting and platelet function outlined in the eligibility criteria for a week prior to the procedure. For the procedure, 1% lidocaine local anesthesia is delivered at the skin (1 to 5 cc) followed by deeper infiltration (about 5 to 10 cc) of 1% lidocaine with or without 1:100,000 epinephrine at the site of each lesion; this will also be used for the core needle biopsy, which will immediately follow the FNA procedure. When more than one eligible lesion is visible in the same patient, one random lesion will be selected.

NOTE: Buffered lidocaine may be used per investigator preference/institutional standard. Next, the FNA procedure itself is performed by sampling the lesion with a 22 to 25 gauge needle attached to a 5 ml syringe with 2-3 passes through each lesion using ultrasound guidance thus confirming accurate targeting. Additional lidocaine may be given if needed for discomfort. The core needle biopsy using standard of care procedures will then occur.

The standard post biopsy instructions, both verbal and written will be provided to all patients. An ice pack is applied to the breast(s) following the procedure, and the subjects are asked to wear a firm sports bra (alternatively, compression wraps or firm bandages may be used).

In our present study, where 1 FNA procedure consists of 2 to 3 passes of the needle into the suspicious lesion, the mean pain rating is 1-1.5 (on a scale of 10); this is consistent with publications from the University of Kansas and the Johns Hopkins study [21, 22] . We may also use a buffered 1% lidocaine so as to minimize the burning sensation associated with unbuffered lidocaine. With our present technique, we are experiencing an average epithelial cell yield of 10,000 cells, and about 10% of samples contain less than 5,000 cells. DNA methylation studies can be performed with as low as 300 cells.

5.2 Cytomorphology

A slide for cytomorphology, stained with Quik Dip (Mercedes Medical) will be prepared by the Sukumar Lab to be read by Dr. Vandenbussche. Results of the cytopathology review will not be given to subjects enrolled in the study; subjects will obtain results from the core needle biopsy recommended for definitive diagnosis.

5.3 Quantitative and Multiplexed Methylation

DNA will be extracted and analyzed by the Cepheid's Breast Cancer Detection cartridge and QM-MSP as previously described in the laboratory of Dr. Sukumar (10;11). Both the cartridge and QM-MSP permit analysis of multiple methylated genes using the same aliquot of DNA with as few as 300 epithelial cells to provide a quantitative estimate of the level of methylation in each gene that exists in each sample.

The cells will be smeared onto 4 uncharged slides. The slides, in batches of 10, will be stained with Quik-Dip by the Sukumar lab and epithelial cells will be counted. The slides will be taken to the cytopathologist to determine their diagnosis (benign or malignant), the cytopathologist will create a report, deidentify the slide with a lab number and keep the lab personnel blinded to the diagnosis.

Cells on the stained slide will be lysed using a custom lysis buffer. An aliquot of the lysate will be used in the automated methylation assay. The results of the methylation assay will be sent to the cytopathologist to compare with standard histopathology based diagnosis report of the H and E stained section of the tumor.

5.4 STRAT4 subtyping assay

A cell lysate from a second unstained slide will be analyzed in the automated STRAT4 (ER/PR/HER2/Ki67) assay cartridge.

STRAT4 results will be compared to standard estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and Ki67 (ER/PR/HER2/Ki-67) by IHC/FISH performed routinely be the pathlab for comparative accuracy of the markers.

5.5 Additional analyses

In addition, FNA lysate or FFPE sections of the resected lesion or core biopsy will be accessed –

a) To compare the predictions of the FNAs as malignant or benign and gene quantitation by the GeneXpert Cancer Detection cartridge to our known laboratory assay, quantitative multiplex-methylation specific PCR (QM-MSP).

b) To perform the STRAT 4 assays in the cartridge using FFPE section of the core of the same malignant lesion to determine concordance between assays performed on FNAs versus resected or biopsied tissue

5.6 Study design

Because this study represents the first use of FNA samples in the GeneXpert cartridge, it will necessary to establish that: 1) a standard FNA sample yields enough cells to perform the assay, and 2) lock down the decision rule for calling a sample malignant based on DNA methylation levels, before 3) obtaining an unbiased estimate of performance. Accordingly, the study will be divided into pilot, training and test phases, respectively, to accomplish these 3 tasks.

Pilot: FNA specimens from the first 5 malignant and 5 benign tumors will be smeared onto uncharged slides, stained and the epithelial cells will be enumerated to ensure that the procedure results in collection of adequate number of cells (>300). If not, additional passes of the needle (up to 5) will be instituted for the FNAs that follow. The remaining cells, if any, will be stored as slides for future optimization of the assay, if needed.

Training Set: A sample size of 80 cases with invasive disease and 80 controls with benign disease is proposed. With the projected incidence of methylated gene markers in benign disease tissue at higher than threshold values at 10%, we project a specificity of 80%. If needed, we will test other tumor specific genes known to us to improve the panel such that a panel of up to 10 genes will function with >90% specificity.

Test Set: A sample size of 80 cases with invasive disease and 80 controls with benign disease is proposed. . If sensitivity and specificity exceeding 90% is achieved in the training set, we will enroll patients for FNAs for the Test set.

We propose to use DNA methylation as biomarkers in cells obtained by fine needle aspiration in a total of 330 women who have suspicious breast lesions recommended by ultrasound guided core needle biopsy for definitive diagnosis. If needed, IRB approval will be requested for the addition of new patients to compensate for FNAs with sparse or no cells, so that projected numbers are fulfilled.

6. Statistical Considerations

The above study plan will recruit 5 women with benign disease and 5 women with malignant disease to undergo FNA prior to core biopsy in the Pilot phase. 80 evaluable subjects with benign disease and 80 with malignant disease will be recruited for FNA prior to core biopsy in Training phase. An additional 80 women with benign disease and 80 women with malignant disease will be recruited for the Test phase if the criteria for the assay are met in the Training set. If not, the assay will be fine-tuned further in the lab with modifications to increase sensitivity and specificity.

6.1 Primary Objectives

6.1.1 Pilot Phase

The goal of the pilot study is to establish that standard FNA samples contain enough cells to complete the proposed assays. The number of epithelial cells obtained from a typical FNA will be

modeled using a Poisson distribution, with parameters estimated from the first 10 subjects (5 malignant, 5 benign).

If the expected probability of obtaining the required 300 cells is less than 99%, additional passes of the needle will be taken to achieve this goal.

6.1.2 Training Phase

The first step in evaluating the ability of the GeneXpert cartridge to distinguish benign from malignant disease, is to establish a decision rule specific for FNA material in the cartridge. Only gold standard pathology on core biopsy samples is used as a standard for comparison during the training phase; results from FNA cytology will not be considered when determining the decision rule. Clinical considerations lead us to target a sensitivity of at least 0.90. Accordingly, we will fix sensitivity at 0.90, selecting the threshold on cumulative methylation to achieve that level, and reporting the conditional specificity along with confidence intervals.

6.1.3 Test Phase

Both primary objectives require the comparison of cartridge results to binary diagnoses made by the study pathologist, so statistical methods for the two objectives are identical. Cumulative methylation values will be calculated for each sample and disease status predicted according to the rule locked down in the training phase. Sensitivity and specificity will be reported, along with exact binomial confidence intervals.

6.1.4 Subgroup Analysis

This study will include women who were referred for mammography after presenting with symptomatic disease as well as asymptomatic women who were identified in the course of routine screening, and it is possible that the assay performs differently in these two populations. Accordingly, in addition to overall performance, we will evaluate and report performance in each of these populations separately.

6.2 Secondary Objectives

Spearman rank correlation will be used to compare the DNA methylation levels as measured on the cartridge to measurements obtained by QM-MSP. Individual genes will be evaluated, as well as the cumulative methylation index. Likewise, Spearman correlation will be used to compare levels of ER/PR/Her2 and Ki67 as measured by the study pathologist, with those obtained using the cartridge.

6.2.1 Sample Size Considerations

Our sample size considerations are motivated by the need for precise estimates of performance, so that an apparently successful model can be carried forward with confidence. Our current methylated marker panel achieves both sensitivity and specificity higher than 90% for distinguishing between normal/benign and malignant disease when evaluated using QM-MSP, and so sample size requirements are calculated at that level. At the proposed sample size, at a sensitivity and specificity of 90%, the estimation errors for the conditional specificity are controlled at +/-10%.

In addition to 10 pilot patients, we will recruit 80 subjects in each group, in each of the two major phases (training and test). This number was selected to control the precision of the confidence intervals on sensitivity and specificity. Specifically, with a sample size of 80 in each group, and sens/spec of 90%, the performance can be estimated to within 10% percentage points (lower, 95% confidence bound =80%), a level of confidence we feel is appropriate for this study.

We estimate that 40%-50% of suspicious lesions will turn out to have malignant disease after path exam of the core biopsy, in which case the limiting factor in obtaining the necessary 330 lesions will be collecting 165 malignant cases. Assuming the lower value of 40% malignancy, we expect to have to enroll 165/0.4=413 patients in order to obtain at least the required 165 cases. At the more extreme ends of the sampling distribution, there is a 10% chance that we can obtain the necessary cases in as few as 382 enrollments, and conversely a 10% chance of falling short even with 447 enrollments . Accordingly, after enrolling 382 subjects we will calculate rates of malignant and benign disease, and estimate total enrollment requirements, and continue enrollment as needed.

7. Adverse Event Reporting

Subjects will be contacted by phone 1-2 days post-FNA and core biopsy to assess adverse events. There is some complexity in this reporting as a core needle biopsy will immediately follow the FNA. The cause, FNA vs core biopsy, of the AE will be inseparable; therefore, determining which process led to the AE will be impossible.

Subjects will also be contacted by phone approximately 10-14 days post-FNA to assess adverse events. Bruising that is still present after 14 days will be considered an AE. If a subject reports bruising at 14 days, we will ask her to be seen by the breast imagers for assessment. A postFNA/biopsy hematoma that requires surgical evaluation will be considered a severe adverse event
(SAE). All adverse events will be recorded and reported to the Clinical Research Office as well as to the IRBs as required by guidelines for adverse event reporting.

8. Records To Be Kept

In addition to the hospital chart, a separate patient study folder will be kept which will include the patient's signed, dated informed consent document.

9. Pathology Requirements

For laboratory/pathology samples:

Laboratory samples will be collected in the clinic by Dr. Susan Harvey or their qualified investigators at Johns Hopkins Imaging at Green Spring. Cytopathology will be assessed by Dr. Vandenbussche and a laboratory identifier will be provided. The Sukumar lab will measure methylation in all samples.

10. Data And Safety Monitoring Plan

This trial will be conducted in accordance with the Data and Safety Monitoring Plan (DMSP) of the Johns Hopkins University's Sidney Kimmel Comprehensive Cancer Center.

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