The Early Detection Research Network (EDRN) Standard Operating Procedure (SOP) For Selection of Archived FFPE Blocks to Support the Benign Breast Disease Project

FINAL

Version 1.0

05-07-2014

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The EDRN Standard Operating Procedure for Selection of archived formalin fixed paraffin embedded specimens of benign conditions of the breast

PURPOSE

To provide a standard operating procedure for identification, selection and retrieval of archived blocks in two large integrated community based health care systems, the Geisinger Health Care System in Pennsylvania and the Henry Ford Health System in Michigan. Processing of tissues from CVCs at Northwestern University, Duke University and Fox Chase Cancer Center for pre-validation studies will be undertaken with the same protocol described here for tissue processing (see Appendix 1).

SCOPE

This procedure applies to identification of the cohort and of cases and controls from the cohort of women with the diagnosis of benign breast conditions, and selection of formalin fixed paraffin embedded blocks.

ROLES AND RESPONSIBILITIES

The SOP applies to all personnel in the designated clinical sites of the EDRN breast working group.

COHORT ASSEMBLY

Inclusion Criteria

1. Diagnosis of benign breast condition (BBC) between January 1, 1994 and December 31, 2005
2. A recipient of health services at the Geisinger Health System or at the Henry Ford Health System at the time of the diagnosis and treatment of the BBC
3. A minimum of 6 months stay with the health care system post their initial diagnosis of BBC
4. Age 40 - 70 years at the time of BBC diagnosis
5. Female Gender

Exclusion Criteria

1. Male Gender
2. Age > 70 or age < 40 at the time of the diagnosis of BBC
3. Previous history of malignancy of the breast or other organ sites except for squamous or basal cell carcinoma of the skin
4. Women whose radio-graphically detected benign lesions were confirmed as lesions of skins or complex nevus by pathologic diagnostic criteria
5. Women whose radio-graphically detected benign lesions were confirmed as secondary to autoimmune disorders, i.e. Sjogren Disease or lupus
6. History of exposure to medical radiation to chest area
7. Referred to the Geisinger Health System or to the Henry Ford Health System
8. Breast cancer diagnosed within 6 months of the diagnosis of BBC
9. Prior mastectomy for CIS or invasive carcinoma.

Cohort Minimum Data Elements (GHS and Henry Ford)

- Patient identifier
- Age at diagnosis
- Gender
- Race
- Date of BBD diagnosis
- BBD side (Left, Right, Both, Not Specified)
- BBD related procedure (Core Biopsy, non-core biopsy, Implant Removal, Lumpectomy, Reduction Mammoplasty)
- Date of biopsy or other procedure
- BBD histology: Microscopic Descriptions
- Availability of BBD diagnostic slides

- Availability of BBD tissue block
- Atypia present : yes/no
- Subsequent cancer and date
- Availability of cancer diagnostic slides
- Availability of cancer tissue block
- Subsequent cancer: in situ, histology, ER, PR, HER2, Grade, Stage (T,N,M)
- Date of last contact

SELECTION OF CASES

Case Definition:

Any member of the cohort diagnosed with a subsequent breast cancer, either in situ or invasive, not earlier than 6 months after her initial diagnosis of benign breast condition and for whom BBD tissue is available. The primary analysis will use invasive cancer cases only.

Exclusion Criteria:

1. BBD tissue block unavailable
2. Cancer diagnosis <=6 months after BBD diagnosis
3. Breast is not the primary site of cancer

Procedures for Identifying Cancer Cases

- Data Sources : Pathology Data Warehouse (Co-Path); Clinical Decision Intelligence System (CDIS); Electronic Medical Record System (EPIC)
- Using MRNs, Link the data from Pathology Data Warehouse to CDIS/Tumor Registry
- From Tumor Registry/CDIS data identify women with history of breast malignancies
- Exclude women from the cohort if cancer occurred before BBD
- Include women as cases if breast cancer occurred > 6 months after BBD and it was not secondary to another cancer

SELECTION OF CONTROLS

For each case diagnosed with cancer at T months after their BBD diagnosis and for whom BBD tissue is available, using the cohort database provided by the study site, the DMCC will provide an ordered list of up to 20 possible matched controls with pertinent data items listed. This list will consist of subjects in the cohort satisfying the following criteria:

i. Not already selected as a control for another case
ii. From the same study site as the case: GHS or Henry Ford
iii. Date of last contact exceeding T months after BBD
iv. Without breast cancer at T months after BBD
v. matched with the case on presence of atypia
vi. matched on BBD related procedure: core biopsy; other biopsy; other procedure(eg implant removal, reduction mammoplasty, unknown)
vii. matched on race (at Henry Ford site; GHS is 98% white)
viii. matched on age: within 5 years
ix. matched on date of biopsy: within 2.5 years

Starting at the top of the ordered list of potential controls, the study site will document the following until 2 eligible matched controls are identified.

a) Availability of BBD tissue block
b) BBD histology category matched with the case: non-proliferative or proliferative or atypia
c) for controls diagnosed with BBD with atypia determine if the control is matched with the case on participation in the STAR trial and on same study arm if applicable

Tissue blocks will be processed for each case and the 2 matched controls.

PATHOLOGIC REVIEW
As there is marked interobserver variability in the pathologic interpretation of proliferative and especially atypical breast lesions, all presumptive cases and controls require review by the local study pathologist. If the original diagnosis is confirmed, the sample can then be processed as outlined below. If there is disagreement, the slides should be additionally interpreted by the central study pathologist (majority diagnosis, best of 3).

Data Elements for the Cases and Controls

Using the VSIMS data key entry system detailed clinical, pathology and outcome information will be collected for all cases and controls contributing tissue to the study. The data items are detailed in the forms (Appendix II).

Processing of FFPE Blocks

Procedures for processing the benign breast disease tissue blocks are detailed in Appendix I.

Candidate Biomarkers

The following are candidate biomarkers for risk of invasive cancer after BBD. The sources for these candidates for consideration are indicated with investigator initial (AG Andy Godwin, JM Jeffrey Marks and PC Paul Cairns). Those attributed to Jeffrey Marks are based on a literature search.

- BRCA1 (PC)
- p16 (PC)
- APC (PC)
- RASSF1A (PC)
- HIN1 (PC)
- EZH2 (JM)
- CEACAM6 (AG/JM, Poola)
- MMP1 (JM, Poola)
- TP53 (JM)
- HYAL1(JM, Poola)
- ALDH1 (JM)
- Periplakin, Epiplakin and Desmuslin (AG)
- Vitronectin and alpha-5-integrin (AG)
- IQGAP2 (AG)
- C5, C8G, C9 (AG)
- Mucin1 (AG)
- Glutathione S-transferase Mu1 and Mu3 (AG)
- ALDH16A1 (AG)

Data Analysis Plan

The performance of each biomarker tested will be undertaken in three steps**:**

A. Relative Risks Associated with Biomarker Values
   1. Cox regression will be used. Time measured from BBD diagnosis.
   2. Relative risks beyond those conferred by other predictors including age, family history, and histology will be examined.
   3. Separate models will be fit for Atypia versus UH versus NP if there are sufficient numbers; Analyses will be combined with stratification if appropriate.

B. Capacity for Discrimination between Cases and Controls
   1. Primary comparison groups: subjects who developed invasive breast cancer by 7 years versus controls who are alive and without cancer at 7 years after BBD
   2. Secondary comparison groups: incident DCIS, invasive cancer after 7 years
   3. ROC curves will be used to compare cases with primary controls. Calculations are complicated because they must handle varying follow-up and the quota method of selecting non-cases
   4. Separate ROCs for BBD with and without atypia will be estimated if possible and compared. We will compare ROC curves for different biomarkers
   5. We will develop a combination biomarker score based on markers that appear to perform well using Cox regression. We will calculate its ROC curve and compare with ROC for best individual biomarker

C. Absolute Risk of IBC for Individual Decision Making
   1. Calculate individual risk of IBC .5-7.0 years after BBD with and without biomarker
   2. Compare risk distributions. How many people classified as high risk (>.75%X4.5) with and without the biomarker
   3. Calculate risk distributions for subjects who develop IBC by 7 years (cases) and for subjects alive without IBC by 7years (controls)
   4. Of subjects who develop IBC by 7 years, how many classified as high risk? This is the sensitivity (TPR)
   5. Of subjects who are alive without IBC by 7 years, how many classified as low risk? This is the specificity (1-FPR)
   6. Calculate the standardized net benefit that combines TPR and FPR into a single index
   7. Compare for different biomarkers and for the most discriminating biomarker combination

Statistical Power

A. Minimally Acceptable Performance

The incidence of IBC in women 50-59 years old is about 0.25% per year (SEER). The incidence in women with BBD is approximately 1.6 times that of normal women, 0.40% per year (Hartmann). Women 50-59 years old should have a breast cancer risk of at least 0.774% in order for positive effects of tamoxifen on breast cancer and hip fractures to offset negative effects on endometrial cancer, pulmonary embolism and stroke (Gail, JNCI 2009). Therefore we set the minimally acceptable performance criterion for a biomarker as positive predictive value >= 0.8% per year to ensure that a positive biomarker value carries with it a clinical consequence, namely a recommendation for tamoxifen in 50-59 year old women. This criterion corresponds to requiring that the ratio of sensitivity (TPR) to 1-specificity (FPR) must be at least 2, because (approximately) positive predictive value = population rate*(TPR/FPR).

B. Power Calculations

If FPR = 0.1 is considered acceptable,(i.e. 10% of women who would not get IBC are treated with tamoxifen therapy,) the study must show that the TPR is at least 0.2. Corresponding sample size requirements are shown below in the first 6 rows of the table. Setting the acceptable FPR=0.2, the study must show TPR is at least 0.4, and pertinent sample size requirements are in the bottom 3 rows.

Acceptable FPR	Minimally acceptable TPR	Actual TPR of the biomarker	Case –control ratio	Number of IBC Cases	Number of Controls
0.1	0.2	0.3	1	380	380
0.1	0.2	0.3	.5	280	560
0.1	0.2	0.3	.25	230	920
0.1	0.2	0.4	1	120	120
0.1	0.2	0.4	.5	85	170
0.1	0.2	0.4	.25	65	260
0.2	0.4	0.6	1	120	120

0.2	0.4	0.6	.5	85	170
0.2	0.4	0.6	.25	70	280

The table shows that with biomarker values for (85 cases, 170 controls) or (120 cases, 120 controls) or (70 cases, 280 controls), we are very likely to make a positive conclusion about a marker whose performance level is (FPR=0.2, TPR=0.6) or (FPR=0.1, TPR=0.4). In other words the power is 90% that the lower confidence bound on the ppv will be at least 0.8% per year for such a marker.

C. Proposed Sample Size

We propose to include 270 subjects diagnosed with BBD, 90 cases who developed IBC within .5-7 years and 180 matched non-cases. If we cannot ascertain tissue for 90 cases of IBC within 7 years, but we can ascertain tissue for 70 cases, then we will expand the number of controls to 280.

APPLICABLE REFERENCES, REGULATIONS AND GUIDELINES

1. Pepe MS, Feng Z, Janes H, Bossuyt PM, and Potter JD. Pivotal Evaluation of the Accuracy of a Biomarker Used for Classification for Prediction: Standards for Study Design. J Natl Cancer Inst 2008; 100:1432-38.

2. Ransohoff DF and Gourlay ML. Sources of Bias in Specimens for Research About Molecular Markers for Cancer. J Clin Oncol 2009; 28:698-704.

APPENDIX I

Standard Operating Procedure (SOP) For Processing of FFPE Blocks to Support the Benign Breast Disease (BBD) Study

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PURPOSE
To provide a standard operating procedure for tissue sectioning and mounting of samples for biomarkers studies of BBD.

SCOPE
This procedure applies to sectioning, mounting, and storing of archival FFPE tissue sample section to support the EDRN biomarker studies.

ROLES AND RESPONSIBILITIES
The SOP applies to all personnel from the EDRN breast working group who are responsible for sectioning tissue preserved in paraffin blocks.

MATERIALS, EQUIPMENT AND FORMS
The materials, equipment and forms listed below are recommendations only and may be substituted by alternative/equivalent products more suitable for the site-specific task or procedure.

Materials and Equipment Materials and Equipment (Site Specific)

- Solvent resistant markers, ink, pencils, and pens
- Microscope
- Microtome
- Hot water bath
- Microtome blades
- Fine tipped paint brush
- Fine tipped tissue separator
- Appropriate labels for slides (provided by DMCC*: one label for FFPE block and 25 serial labels for slides along with corresponding worksheets to be entered into VSIMS)
- Labeled glass slides
- Tray to hold slides
- Ice tray
- Oven
- Labeled electrostatically charged slides (such as SurgiPath Plus slides)
- Film for sealing slide boxes such as Parafilm
- Slide storage boxes (labeled with DMCC Box#)
- Harris Haematoxylin (filtered)
- Eosin

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Health/Safety Warning
When working with potentially hazardous materials, follow EPA, OSHA, and other specific health and safety procedures. Be prepared in case of emergency (e.g., telephone numbers, first aid kit). Personnel should wear an apron or lab coat, gloves and safety glasses when handling hazardous chemicals.

PROCEDURES
This procedure is intended to ensure that tissue samples preserved for research studies are sectioned in a safe and consistent manner while eliminating the risks of contamination and loss of molecular and structural

integrity. It also ensures rationing of the tissue blocks associated for each case for multiple assays and projects and maintenance of the block orientation. Consistency in procedure is important for obtaining comparable and reliable test results.

These procedures also outline minimum steps that should be followed to ensure that tissue samples collected, stored and distributed are of sufficient morphological and molecular caliber to meet the research needs of the investigators.

Selection of blocks

- For each case, identify the "most significant" category of BBD (atypical lesions > proliferative lesions without atypia > nonproliferative lesions). Try to sample all lesions in that category (e.g., adenosis, apocrine change and simple fibroadenoma). Ideally the different lesions should be captured in one paraffin block, but multiple blocks per case may be selected to capture all relevant lesions.
- For each block one BBD Pathology Form should be filled out.

Sectioning Formalin Fixed Paraffin Embedded Tissue

- Treat all tissue as potentially infectious.
- Since some sections will be used for nucleic acid studies, whenever possible all instruments and equipment should be pre-cleaned and wiped down with RNAse-away before and between each specimen. Gloves must be worn. Molecular grade water is recommend to be used for floating sections for RNA extraction.
- Sectioning is performed by the laboratory or histology technician/technologist or personnel trained to use a microtome and cut histological sections.
- Have materials and equipment ready. Have as many slides as needed labeled and ready.
- Pre-cool paraffin blocks, tissue side down, on a tray of ice. In some cases this may facilitate sectioning. Using a steel microtome knife or disposable blade cut 25 sections/block at 5 microns/section for histological and nucleic acid extraction purposes.
- If there is insufficient tissue for complete sectioning, only cut as many sections as possible without depleting the tissue. Note, tissue sections without the actual lesion may still be important to study "field effects" of the biomarkers, so whenever possible prepare 25 slides.
- Label slides serially from 1 (top) to 25 (bottom) using the labels provided by the DMCC, indicate on worksheet which slides were created and whether or not the slide is designated H&E.
- Dry paraffin sections at room temperature overnight.
- Sections 1, 13 and 25 are to be stained with H&E using standard procedures.
- Since benign breast lesions may be very small, it is possible that they may not be present on deeper levels. In that case their absence should be noted by the reviewing pathologist.
- The unstained sections are stored at -20ºC or -80ºC prior to shipping to the centralized EDRN pathology core (University of Kansas Medical Center*) in slide holder boxes.
- The FFPE block needs to be associated to the Participant ID in VSIMS (Validation Study Information Management System - a web-based data entry and specimen tracking system provided by the EDRN:DMCC). The individual slides need to be entered in VSIMS and then the slide box needs to be shipped to KUMC (to address provided below). Some sites may choose to store the slide sections locally until requested to provide for biomarker analysis. Prior to any samples being shipped for analysis either the collection site or the KUMC Central Pathology Lab will need to re-label the slides with a unique ID provided by the DMCC and associate that ID to the serial ID in VSIMS prior to shipment.
    - Example: Site ID XXX has entered Clinical Information for Participant ID XXX in VSIMS. Collection site will associate Participant ID XXX to FFPE ID.
    - BBD FFPE IDs will start with Protocol ID 331 followed by 5 digit number. Each Slide ID will include the Protocol ID 331 followed by the same 5 digit number as the FFPE ID and appended with 1, 2, 3, 4, etc… serially.

- Note to biomarker validation sites. Prior to any immunohistochemical staining, the slides should be heated at 60 degrees C for 40 min using either an oven or warming tray.

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Quality Assessment – General Considerations for Section Review

- At a minimum, assessment must consist of morphologic review of tissue sections.
- Use researcher feedback about section quality to refine practices and guide evolution of Quality Control procedures.

Quality Assessment – Issues Concerning Quality of Sections

- Make sure that representative tissue remains in the block after sections are cut for an assay. Do not deplete paraffin blocks.
- Since some sections are intended for Polymerase Chain Reaction (PCR)-based molecular studies make sure that all attempts are made to eliminate or minimize nucleic acid contamination from equipment or other samples.
- Ensure that section thickness is consistent and appropriate for intended use.
- Ensure that sections are not scored or torn by the microtome knife as this will obscure microscopic observation and may cause uneven staining or bias assay results.
- Ensure that thin sections are placed on electrostatically charged slides to avoid loss of the section during the immunohistochemical assay.
- Ensure that paraffin sections are stored and shipped under appropriate conditions and temperatures.

Quality Assessment – General Sectioning Regimen for QA Safeguards

The use of this schema is recommended to ensure that representative sections from a sectioned block are kept for quality assessment purposes. Perform these steps at the time the block is being sectioned for a research application.

- Ensure that a representative Hematoxylin and Eosin (H&E) section is retained from the block within the biobank.
- If no H&E is available from the last sectioning of the block retain a “top” section for H&E review.
- If many sections are taken from a block, it may be useful to retain “intermediate” sections from the tissue block for H&E review.
- Label sections serially. Also record the date the section is cut on the worksheet.

APPLICABLE REFERENCES, REGULATIONS AND GUIDELINES

1. Declaration of Helsinki
http://www.wma.net/en/30publications/10policies/b3/index.html
2. Tri-Council Policy Statement 2; Ethical Conduct for Research Involving Humans; Medical Research Council of Canada; Natural Sciences and Engineering Council of Canada; Social Sciences and Humanities Research Council of Canada, December 2010.
http://www.pre.ethics.gc.ca/eng/policy-politique/initiatives/tcps2-eptc2/Default/
3. Human Tissue and Biological Samples for use in Research. Operational and Ethical Guidelines. Medical Research Council Ethics
http://www.mrc.ac.uk/Utilities/Documentrecord/index.htm?d=MRC002420
4. Best Practices for Repositories I. Collection, Storage and Retrieval of Human Biological Materials for Research. International Society for Biological and Environmental Repositories (ISBER).
http://www.isber.org/Search/search.asp?zoom_query=best+practices+for+repositories
5. US National Biospecimen Network Blueprint
http://biospecimens.cancer.gov/resources/publications/reports/nbn.asp

6. Jewell, S. et al. 2002, Analysis of the Molecular Quality of Human Tissues, an experience from the Cooperative Human Tissue Network. Am. J. Clin. Pathol. 118:733-741.
7. Guideline – Fresh Tissue Working Group of BIG and NCI breast cancer Cooperative Groups
8. SOP No.3 (Draft 1). November 15, 2005. Standard Tissue Sectioning. NCIC CTG. Ontario.
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10. Recommendations of FFPE Working Group of BIG and North American breast Cancer Groups.

APPENDIX II

Data Collection Forms

Separate PDF