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**Microgenomic Approaches to Identify Clinically
Relevant Gene Signatures that Discriminate
Histologic Types of Breast Carcinomas**

By

Sean Thomas Butterbaugh

**Submitted in partial fulfillment of the requirements
for Graduation *summa cum laude***

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May, 2014

*University of Louisville College of Arts & Sciences
Honors Thesis*

**MICROGENOMIC APPROACHES TO IDENTIFY CLINICALLY
RELEVANT GENE SIGNATURES THAT DISCRIMINATE
HISTOLOGIC TYPES OF BREAST CARCINOMAS**

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ABSTRACT

Background: Breast cancer presents itself in a variety of histologic types, and the two most common types are invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC). Based on comparative genomic hybridization (CGH) analyses, ILC is more closely related to low grade IDC than it is to intermediate and high grade IDC. Results from the BIG 1-98 trial demonstrate that post-menopausal women who are affected with estrogen receptor positive (ER+) ILC or luminal B (high grade) IDC experience a greater magnitude of benefit when they are treated with the aromatase inhibitor (AI) letrozole compared to treatment with the antiestrogen tamoxifen. To contrast, it has been found in the same trial that women affected with luminal A (low grade) IDC experience more benefit when treated with tamoxifen when compared to letrozole. It is therefore imperative to accurately distinguish low grade IDC from ILC considering their varying responses to adjuvant treatment. Despite genetic evidence suggesting a close relationship between ILC and low grade IDC, a clinically relevant gene set underlying a tumor's biologic responsiveness to letrozole likely exists. The goal of this study is to use microgenomics to identify a clinically relevant candidate gene set that would discriminate between ILC and low grade IDC rather than relying solely on histomorphology and/or immunohistochemistry for the pathologic diagnosis, especially when conventional tests are conflicting.

Methods: Using 247 de-identified human breast carcinoma biopsies collected under standardized, stringent conditions, total RNA was extracted from carcinoma cells procured by laser capture microdissection to perform microarray analyses of approximately 22,000 genes to identify expression signatures associated with breast cancer characteristics. Of the 247 LCM-procured samples, 14 were ER+ ILC, 9 were ER+ low grade IDC, and 43 were ER+ high grade IDC. The other 181 samples were either ER- or of another cancer type other than ILC and IDC. Candidate genes were selected by identifying those that were differentially expressed between ILC and low grade IDC (luminal A) and at the same time, had similar expression levels between ILC and high grade IDC (luminal B). qPCR analyses of whole tissue samples were then utilized to validate the selected gene set.

Results: Comparison of microarray data from hormone receptor positive tumors yielded 299 probes that were differentially expressed ($p < 0.01$) between ILC and low grade IDC (luminal A), and 99 of these probes were not differentially expressed ($p > 0.01$) between ILC and high grade IDC (luminal B). 11 of these 99 genes were initially chosen for further investigation by performing qPCR on whole tissue samples from 21 ILC, 19 low grade IDC and 19 high grade IDC tumors. Our initial analysis revealed expression of the gene coding for heparin-binding EGF like growth factor (HBEGF) and collapsin response mediator protein 1 (CRMP1) may be potential markers for differentiating between ILC and low grade (luminal A) IDC.

I. INTRODUCTION

Overview of Breast Cancer

Data accumulated in the SEER Database and in the long-term records of the American Cancer Society indicate that one in eight women will present with an invasive breast carcinoma at some point in her life. An estimated 232,340 new cases of breast cancer are expected to be reported in 2013 in the United States and more than 39,000 women are predicted to die from this disease. Although the death rates from breast cancer have been decreasing, the incidence rates have been increasing since 1975 (1). Postmenopausal women are at a higher risk of developing breast cancer compared to premenopausal women (2).

Pathology

When a breast tumor is excised from a patient, it is sent to pathology for diagnosis. Pathologic diagnosis for invasive carcinoma includes, but is not limited to, histologic type, histologic grade and estrogen receptor, progesterone receptor and HER2 status. The results provided by pathology guide patient management for selection of appropriate adjuvant therapy (3).

The most common histologic type of invasive breast carcinoma is invasive ductal carcinoma (IDC), no special type. IDC accounts for 70-75% of all invasive breast carcinomas. The second most common histologic type is invasive lobular carcinoma (ILC), which accounts for 10% of all invasive breast carcinomas (4). The distinction between IDC and ILC is determined on hematoxylin and eosin stain (H&E), where IDC characteristically infiltrates as cohesive solid nests and tubules, and ILC characteristically infiltrates as single cells in single file. The differing appearance between these two histologic types is due to the presence or absence of E-Cadherin which is a membrane adhesion molecule (5). IDC expresses functioning E-Cadherin at its cell membrane surface allowing for adhesion among tumor cells resulting in the ability to form cohesive structures such as tubules, whereas ILC lacks functional E-Cadherin and therefore exists as single cells infiltrating through stroma. Occasionally invasive carcinoma on H&E section can show variable histologic features with areas of the tumor demonstrating single cells in single file and other areas of the tumor demonstrating cohesive nests and tubule formation. Adjunct immunohistochemical studies using antibodies to E-Cadherin can be used to support the histologic diagnosis (6).

Typically, a lack of E-Cadherin immunostaining supports the diagnosis of ILC and the presence of membranous staining with E-Cadherin supports the diagnosis of IDC (3). Although helpful in the majority of cases, E-Cadherin immunohistochemistry can be positive in 5-15% of ILC cases and occasionally low grade carcinomas demonstrating ductal features on H&E can be negative for E-Cadherin, resulting in possible misclassification of the histologic type (6). In the past, the distinction between ILC and IDC was not imperative for treatment purposes since IDC and ILC were treated the same, however with the recent results of the BIG 1-98 trial, discriminating between IDC and ILC will decide whether the patient will benefit more from Tamoxifen versus an aromatase inhibitor, which will be discussed further throughout this Thesis (25). As such, it is clinically imperative to be able to distinguish between ILC and IDC due to variable responses to hormonal therapies as well as assessment of survival prognosis.

Figure 1. Challenges in diagnosing invasive lobular carcinoma versus invasive ductal carcinoma of the breast. (A) Invasive carcinoma illustrating the formation of tubules (encircled). The same invasive carcinoma in (A) stains negatively for E-cadherin with the internal positive control shown (arrow) (B). (C) Invasive carcinoma showing infiltration as single cells (arrow) and in single file (*), which is E-Cadherin positive (D).

Grading

The grading of a breast tumor is a clinically relevant prognostic factor that measures the degree of differentiation in the carcinoma. The Nottingham grading system considers three criteria: tubule formation, nuclear pleomorphism and mitotic counts (7). The scale ranges from 1 to 3 with Grade 1 carcinomas being well-differentiated and Grade 3 lesions appearing as poorly-differentiated. Patients exhibiting Grade 1 carcinomas have the most favorable prognosis in regard to overall survival (OS) and disease free survival (DFS) while those patients with Grade 3 cancers have the poorest predicted outcomes (8). The study population consisted of de-identified patients exhibiting either IDC or ILC on which there are microarray expression data of 22,000 genes using LCM-procured breast carcinoma cells (9, 10). Clinical features of this patient population were assessed to ensure they represent the population at large of breast cancers. We observed that there was a noticeable difference in patient survival, both OS and DFS, between those exhibiting low grade (Grade 1, n=13) compared to high grade

(Grade 3, n=85) carcinomas (Fig. 2). The risk of recurrence and survival are very different among the various grades which is extremely important considering that grading has been shown to be an independent predictor of survival and recurrence in invasive carcinomas (8). It should also be noted that there was a difference in DFS and OS of patients with Grade 1 IDC compared to ILC and properly distinguishing between the two may serve as a predictor of survival and an indicator of aggressiveness, which would be useful when treating the disease.

Figure 2. Kaplan-Meier survival curves of patients with ILC and graded IDC among the population with microarray data. (A) Disease Free Survival (DFS), (B) Overall Survival (OS)

Conventional Biomarkers: ER, PR, and HER-2/neu

Estrogen Receptor

The estrogen receptor (ER) is a nuclear steroid hormone receptor protein whose presence, or lack thereof, is used as a prognostic factor as well as a predictive biomarker correlating with responses to endocrine therapies for advanced breast carcinomas (11, 12). As a prognostic factor, it along with the progesterin receptor (PR), serve as independent predictors of breast cancer survival (13). Currently, there are two widely acknowledged estrogen receptors, ER α and ER β , and they have been found to be regularly expressed in different tissues in the body. ER α is expressed in the major female organs, (including the mammary gland), the hypothalamus, and bone while ER β is expressed in the ovary, major male organs, and parts of the central nervous system (14). It is the ER α protein that is useful as a predictive factor in hormone treatment responsiveness, with ER+ invasive breast tumors displaying a more favorable response to adjuvant treatment with antiestrogens such as Tamoxifen than ER- carcinomas (15, 16). Beyond simply functioning as a predictive indicator, the ER in breast cancer has become a target for developing highly specialized endocrine therapies, including both antiestrogens and aromatase inhibitors, which will be discussed later.

Progestin Receptor

The progestin receptor (PR) is a nuclear steroid hormone receptor that is regulated by the ER (11, 12). The presence of PR in a tissue is thought to indicate an active ER in the same cells and thus can be used as a predictive factor for more accurate responses to endocrine therapies in ER+ breast tumors (11, 12, 17). Roughly 5% of breast carcinomas are ER-/PR+ but they often respond to hormone therapies (15, 16, 18).

Sex-Hormone Receptor Analyses

There are two major methods used in quantifying steroid receptor proteins:

The first, the ligand-binding assay (LBA), which is also known as the multipoint titration assay (MTA), detects the presence of steroid receptors by using radiolabeled ligands (e.g. [³H]estradiol-17 β) that bind specifically to estrogen or progesterone receptors. This particular assay quantifies the concentration of receptor proteins, which is expressed as the specific binding capacity measured in femtomoles of radio-labeled ligand bound per milligram of cytosol protein (fmol/mg). Using the LBA, the dissociation constant (K_d value) also may be estimated, which measures the binding affinity of the labeled ligand to its receptor. Using this type of assay, the cutoff value indicating ER+ or PR+ in tissues has been set at ≥ 10 fmol/mg by the FDA (11, 12). However, this assay is not specific to ER α and may also be used to detect ER β with ligands exhibiting estrogenic activities (19). The second major method involves the utilization of antibodies to detect epitopes that are specific to each of the receptor proteins. Because the antibodies are unique to each receptor protein, the presence of ER α and ER β may be determined separately. Two assays that use this method are the enzyme immunoassay (EIA) (20) and the enzyme-linked immunoassay (ELISA) (11, 12). Because the EIA and ELISA techniques specifically detect a domain on the receptor protein, their results are not affected by endogenous or exogenous steroid hormones. Both EIA and ELISA utilize a spectrophotometer to measure the intensity of the labeled colored product, which allows accurate quantification of the receptor proteins.

IHC analysis (21) also use antibodies specific to the receptor proteins; however the assay only provides semi-quantitative results (22). However, a problem with IHC is that the interpretation of the degree of staining by the labeled antibodies is subjective (highly operator dependent) due to variable stain intensities due to unstandardized conditions that have only been corrected recently (22). Thus the results of this assay are only semi-quantitative, and were not used in the studies described in this thesis. Since the ER and PR proteins arise from the translation of their cognate mRNA molecules, real-time reverse transcription polymerase chain reaction (RT-PCR) is a reliable technique for measuring gene expression. This assay determines the level of mRNA for the genes coding for ER (ESR1) and PR (PGR) in a sample and compares this to a control gene, producing a relative expression level (23). RT-PCR is able to specifically look for the gene coding for ER α or ER β as well as any other known gene.

Therapeutic Treatments for ER+ Invasive Carcinomas

The complex of female sex hormone or its mimic with the estrogen receptor protein is required to initiate a response in both normal and carcinoma cells. One of the responses of the ER signaling pathway is the production of the progesterin receptor protein. Thus, the hormone receptor status of normal breast tissue cells and breast cancer cells is physiologically important because estrogens and progestins stimulate differentiation and proliferation in both types of breast tissue. Therefore, if a breast cancer biopsy is ER+, its growth may be controlled by several therapeutic approaches following the surgical removal of the primary carcinoma. One of the therapeutic approaches involves blocking the ability of the ER protein to bind native estrogen molecules in a cell, thus inhibiting its action. This type of treatment utilizes manmade drugs called antiestrogens (also called SERMs, Selective Estrogen Receptor Modulators), such as the drug Tamoxifen (15, 16, 24).

A second class of therapeutic agents reduces the amount of estrogen produced and circulating in a female patient's body by inhibiting the enzymatic conversion of androgen building blocks into estrogens. The enzyme promoting this conversion of a weak male sex hormone into an estrogen in adipose tissues is called an aromatase, and the manmade drugs that inhibit the enzymatic activity are called aromatase inhibitors (AI). An example of a currently used AI is Letrozole (25). AIs apparently do not block the production of estrogens in the ovaries due to the high level of production and thus the second therapeutic approach appears to be most effective in post-menopausal women since their ovaries are no longer producing estrogen.

Although AIs are most effective in post-menopausal breast cancer patients, it has been found that different types of invasive breast carcinomas have varied responses to Letrozole. For example, patients with low grade IDC (luminal A-like) exhibit a trend toward lower overall survival than women with ILC when these patients were treated with Letrozole (25). This suggests there is a molecular basis for the differences observed in the therapeutic responses of the two histologic types of breast carcinomas.

HER-2/neu Oncoprotein

HER-2/neu is a transmembrane glycoprotein that is a member of the human epidermal growth factor receptor (HER, or EGFR) family and was originally identified in neuroglioblastoma of rats, hence *neu*. The other members of the EGFR family are HER-1, HER-3, and HER-4. HER-2 has no known ligand but acts by heterodimerizing with another member of the family and the heterodimer becomes active when the internal domain of the other dimer member is phosphorylated via tyrosine kinases (26). Overexpression of HER-2 oncoprotein in breast cancer appears to be associated with poor prognosis (27). Breast carcinomas with overexpression of HER-2 have been shown to have a poorer response to endocrine therapies than tumors with an expression level that is normal to non-cancer cells (28). The expression of the HER-2 protein has a clinical utility in that patients with carcinomas that overexpress it are more likely to respond to therapies that target HER-2 such as the drug Herceptin® (trastuzumab) (29).

HER-2/neu Oncoprotein Analyses

Since HER-2/neu oncoprotein has no known ligand, the manner in which the membrane bound biomarker may be determined utilizes antibody based assays in either EIA or ELISA formats (11, 12). Therefore, the quantified results represent HER-2/neu content in a breast cancer tissue biopsy, but not activity. The current assay for assessing HER-2/neu oncoprotein in tissue biopsies is by IHC (27). However, early studies utilized “home-brew” assays before standardization guidelines were recently issued by the College of American Pathologists (CAP) in association with the American Society of Clinical Oncologists (ASCO) (30). It should be noted that all values used in the studies reported in this thesis were quantified either by EIA or ELISA.

Statement of Problem

It is imperative to be able to properly distinguish between the two histologic types, Low Grade IDC and ILC, and we propose that a set of genomic markers exist within breast carcinoma cells that allow a molecular means of distinguishing them as an aid to the pathologist. Our hypothesis is that each of these histologic types of breast carcinoma, IDC and ILC, may be identified using a molecular signature reflecting a distinct pattern in the expression of certain genes. This set of genomics-based biomarkers will advance the pathologist’s ability to discern IDC from ILC when conventional pathologic parameters are conflicting. Discriminating between Grade 1 IDC and ILC would improve the ability to assess prognosis (*i.e.*, risk of recurrence) as well as aid in selection a treatment regimen more likely to promote a response for breast cancer patients.

II. RELATIONSHIP OF EXPRESSION OF CONVENTIONAL BIOMARKERS IN ILC COMPARED TO IDC

Currently, ER PR and HER-2/neu protein quantities in breast carcinoma tissue biopsies serve as the principal biomarkers for assessing a patient's risk of recurrence and as a predictor of therapeutic response. (11, 12). Therefore, data mining studies were performed using deidentified information in the comprehensive IRB-approved Database to ascertain whether expression of these protein biomarkers is related to the various histologic subtypes and the ability to distinguish ILC from Grade 1 IDC.

Methods and Materials

Estrogen Receptor and Progestin Receptor Levels

ER and PR protein levels were determined previously using either enzyme immunoassay (EIA) or radio-labeled ligand binding assay (LBA) (11, 12, 31) and the results were incorporated into the comprehensive, de-identified Database established by Dr. Wittliff. Briefly, both methods utilized chilled/frozen specimens that were sliced carefully with a scalpel on a Petri dish chilled on a frozen ice pack to maintain receptor integrity and then homogenized with a mass-to-buffer ratio of 100 mg wet weight of tissue per 1.0 ml of 40 mM Tris-HCl buffer, pH 7.4, containing 1.5 mM EDTA, 10% glycerol, 10 mM sodium molybdate, 10 mM monothioglycerol and 1 mM PMSF (12). Extracts were prepared by centrifugation at 100,000 x g for 30 min.

A complete ligand binding assay was comprised of duplicates of six increasing concentrations of radiolabeled [^3H -estradiol-17 β] with and without unlabeled diethylstilbestrol in a titration format (12, 31). Reactions were incubated overnight (12-18 h) at 4°C. Unbound ligand was removed by addition of dextran-coated charcoal, incubated for 15 min, and then centrifuged at 3300 x g for 15 min at 4°C. Supernatant was removed and radioactivity was detected in a liquid scintillation counter. Specific ligand binding capacity, reflecting the receptor level, was expressed as fmol/mg cytosol protein while the resulting apparent dissociation constant (Kd value) determined by Scatchard analysis was expressed as M.

Determination of ER and PR levels by EIA employed a kit formerly distributed by Abbott Laboratories (12, 31) and the results were incorporated into the comprehensive, de-identified Database. This protocol utilized beads coated with Anti-ER monoclonal antibodies, which were incubated with the tissue extracts (12, 31). Unbound materials were aspirated and washed, before the bead-associated receptor protein was incubated with anti-receptor antibodies conjugated with horseradish peroxidase. Color was developed and measured with a spectrometer at a wavelength of 492 nm. The receptor level (mass) was expressed as fmol/mg cytosol protein. Thus each of the ER and PR results represent highly quantified values.

Results and Discussion

Relationships of Biomarker Protein Levels and Breast Cancer Subtypes

The first investigation that was conducted related to the hypothesis was to determine if there was a relationship between the expression of the conventional breast cancer biomarkers and patient/cancer biopsy characteristics. This was accomplished by mining the IRB-approved comprehensive Database. After completing the evaluation of ER, PR, and HER-2/neu oncoprotein individually, assessment of the utility of combining expression of ER and PR protein was performed. As explained earlier, these analyses of sex hormone receptors are routinely performed as a pair by IHC (22). Although, triple negative breast cancer is the subject of many investigations because of the implication for aggressive disease which is difficult to treat (4, 16), the limited number of breast cancer tissue biopsies used in the current study with HER-2/neu results precluded examination in combination with other biomarkers. Furthermore, our genomic studies focused primarily on ER+ breast carcinomas.

Examining data from ER+ tissue samples, no significant difference was found in the ER protein levels between ILC (n=90) and Grade 1 IDC (n=90). The median concentration in ILC using LBA and/or using EIA was 129 fmol/mg. The median protein concentration in Grade 1 IDC using LBA and/or EIA was 150 fmol/mg. When performing a Dunn's multiple comparisons test, the only significant difference ($p<0.05$) in quantified protein concentrations was between Grade 2 IDC and Grade 3 IDC (Fig. 3).

Figure 3. Quantified ER protein levels in different histologic types.

In PR+ tissue samples, there was no significant difference found in the progesterin receptor protein levels between ILC (n=85) and Grade 1 IDC (n=78). The median concentration in ILC using LBA and/or EIA was 161 fmol/mg. The median protein concentration in Grade 1 IDC using LBA and/or EIA was 256 fmol/mg. However, it was found that progesterin receptor protein levels were significantly lower ($p<0.05$) in Grade 3 IDC (n=208) when compared respectively to Grade 1 IDC, Grade 2 IDC (n=235), and ILC (Fig. 4).

Figure 4. Quantified PR protein levels in different histologic types.

A similar type of analysis was performed using the HER-2 oncoprotein results from each of the breast cancer subtypes. The quantified levels of the HER-2/neu oncoprotein also showed no statistically significant difference ($p < 0.05$) among any of the grades of IDC and ILC (Fig. 5).

Figure 5. Quantified HER-2/neu protein levels in different histologic types.

ER and PR protein levels in breast carcinoma biopsies are routinely analyzed as described earlier. To determine if their expression was different in the breast cancer subtypes, ER and PR quantities in breast cancers were analyzed as a function of IDC

grade compared to that in ILC (Fig. 3-5). When analyzing the entire patient population with associated clinical data (n=831), we found that patients presenting with ILC (n=97) and Grade 1 IDC (n=85) have similar expression of ER positivity at 88% and 93%, respectively, while only 44% of Grade 3 IDC (n=350) were positive for ER using established cut-off values (Table 1A).

In regard to progesterin receptor status, ILC (n=97) and Grade 1 IDC (n=85) exhibited similar expression of PR positivity at 84% and 79%, respectively, in the study population while only 55% of Grade 3 IDC (n=350) exhibited PR+ levels (Table 1A). Closer examination of the data revealed that 78.4% of the study population that presented with ILC (n=97) had ER+/PR+ status and 73.9% of Grade 1 IDC (n=73) biopsies were ER+/PR+. A much lower expression of ER+/PR+ status (36.8%) was observed for Grade 3 (n=291) carcinomas (Table 1B). Low grade experiencing higher ER and PR positivity and high grade experiencing the opposite as well as ILC showing similar hormone receptor positivity as low grade IDC is consistent with existing literature (13, 32)

Due to the similar protein expression levels and statuses, ER, PR, and HER-2/neu do not aid in distinguishing ILC from Grade 1 IDC.

A.

ILC (n=97)		Grade 1 IDC (n=85)	
ER	PR	ER	PR
Positive - 85 (88%)	Positive - 81 (84%)	Positive - 79 (93%)	Positive - 67 (79%)
Negative - 12 (12%)	Negative - 16 (16%)	Negative - 6 (7%)	Negative - 18 (21%)

Grade 2 IDC (n=299)		Grade 3 IDC (n=350)	
ER	PR	ER	PR
Positive - 240 (80%)	Positive - 220 (74%)	Positive - 154 (44%)	Positive - 192 (55%)
Negative - 59 (20%)	Negative - 79 (26%)	Negative - 196 (56%)	Negative - 158 (45%)

B.

ILC (n=97)		Grade 1 IDC (n=73)	
ER+/PR+	76 (78.4%)	ER+/PR+	54 (73.9%)
ER+/PR-	9 (9.3%)	ER+/PR-	14 (19.2%)
ER-/PR+	5 (5.2%)	ER-/PR+	4 (5.5%)
ER-/PR-	7 (7.2%)	ER-/PR-	1 (1.4%)

Grade 3 IDC (n=291)	
ER+/PR+	107 (36.8%)
ER+/PR-	21 (7.2%)
ER-/PR+	48 (16.5%)
ER-/PR-	115 (39.5%)

Table 1. ER and PR Statuses among Entire Patient Population. (A) ER and PR statuses among varying histologic types. **(B)** ER/PR statuses among varying histologic types.

Relationships of Biomarker Gene Expression Levels and Breast Cancer Subtypes

In order for a biomarker protein to be synthesized in a cell, expression of the cognate gene must occur and there must be fidelity in the transcription of the gene and the translation of the mRNA. This occurs routinely in normal cells. However, the molecular basis of gene and protein expressions in carcinoma cells may be discordant because of errors at various steps in the process. Since the levels of the ER, PR and HER-2 proteins in breast cancer tissue biopsies were not related to histologic subtypes, the expressions of the receptor genes were examined using results in the comprehensive Database.

Expression of the genes that code for the estrogen (ESR1) and progesterin (PGR) receptor proteins and the HER-2/neu (ERBB2) protein (Fig. 6) were ascertained by analyzing microarray data of breast carcinoma cells from 247 tissue biopsies (168 of those cancer specimens were either IDC or ILC) that were obtained via laser-capture microdissection (LCM) (9, 10) as will be discussed further in Chapter III.

Examination of the microarray data clearly indicated that ESR1 expression was not significantly different ($p < 0.05$) between Grade 1 IDC ($n=13$) and ILC ($n=15$). It is worth noting that the relative gene expression in Grade 3 IDC ($n=85$) was significantly lower ($p < 0.05$) than all other tumor histologic types which supports previous evidence of a lower percentage of ER+ tumors being exhibited in Grade 3 IDC (Fig. 6A) (13).

Using the same extensive Database, PGR expression also was not significantly different ($p < 0.05$) in LCM procured breast carcinoma cells from Grade 1 IDC ($n=13$) compared to those from ILC ($n=15$). The gene expression in Grade 3 IDC ($n=85$) was significantly lower ($p < 0.05$) than that of the other tumor histologic types which again supports previous reports that the lower percentage of PR+ tumors are Grade 3 IDC (Fig. 6B) (13).

Using similar analyses for ERBB2, the relative expression levels of this gene were not significantly different ($p < 0.05$) among any of the breast carcinoma subtypes examined in this study (Fig. 6C).

Figure 6. Relative gene expression levels from microarray data (N=168) of genes coding for the conventional biomarkers from patients were analyzed.

In summary, careful analyses of quantified results from the comprehensive Database indicated there were no significant differences between Grade 1 IDC and ILC with regard to expression of the biomarkers ER, PR, and HER-2 oncoprotein. This was observed for the frequency distribution of positive/negative statuses, receptor protein concentration, and relative gene expression. Thus, neither the expression of the biomarkers proteins nor their genes serve as appropriate diagnostic tools for discriminating between Grade 1 IDC and ILC when conventional pathologic tests are conflicting. It is necessary to identify new biomarkers to accomplish this goal. This clinically relevant question was then approached using microgenomics to identify a gene signature that may be used to differentiate between these two histologic types in order to improve diagnosis and therapy selection.

III. IDENTIFICATION AND VALIDATION OF POTENTIAL GENE SIGNATURES BY COMPARING EXPRESSION IN ILC AND IDC

Methods and Materials

Using an IRB-approved Database and Biorepository composed of de-identified specimens previously collected under stringent conditions (12) for clinical assays of estrogen (ER) and progesterin receptors (PR), tissue sections of primary invasive ductal carcinomas obtained from 1988-1996 were examined using REMARK criteria (33). Patients were treated with the standard of care at the time of diagnosis. Tissue-based properties (e.g., pathology, grade, size, and tumor marker expression) and patient-related characteristics (e.g., age, race, smoking status, menopausal status, stage, and nodal status) were utilized to determine relationships between gene expression and clinical parameters.

Carcinoma cells were procured from tissue sections of 247 frozen breast cancer tissue specimens using the PixCell Ite (Arcturus) laser capture microdissection (LCM) instrument and workstation using protocols established in our laboratory (cf (9, 10, 10)). A retrospective analysis of gene expression was also performed on intact frozen tissues from 233 biopsies of invasive ductal carcinoma (Table 3). Tissue sections utilized for these analyses contained a median of 60% breast carcinoma cells (range of 10-95%) and 25% stromal cells (range of 5-65%).

Gene Selection

Gene candidates for identifying a molecular signature were selected by mining the results from microarray analyses of LCM-procured carcinoma cells from 247 de-identified human breast carcinoma biopsies. Of the 247 carcinoma specimens (disregarding ER/PR statuses), 16 were ILC, 13 were low grade IDC, 55 were intermediate grade IDC and 85 were high grade IDC (Fig. 7).

Initial analyses identified 1267 probes that were differentially expressed ($p < 0.01$) between cell samples from patients with IDC compared to those with ILC, regardless of ER/PR status. Gene expression levels in breast carcinoma cells from patients with low grade IDC compared to those with ILC yielded 200 sequences that were differentially expressed ($p < 0.01$). When comparing high grade IDC to ILC, 149 of these genes were not differentially expressed (Fig. 7).

Similar analyses were performed using only the microarray results from patients with ER+/PR+ cancers ($n=107$). Gene expression levels in carcinoma cells from patients with low grade IDC compared to ILC yielded 299 probe sequences ($p < 0.01$). 99 of these genes were not differentially expressed when comparing high grade IDC to ILC ($p > 0.01$). 15 genes were the same in the analysis of low grade IDC compared to ILC regardless of ER/PR status and in the same analysis with only ER/PR+ patient samples indicating that this gene set may serve as a panel of gene candidates. Of the 15 probes sequences, 11 genes were investigated further by qPCR (Fig. 7).

Figure 7. Flow chart illustrating the strategy for selecting gene candidates using the microarray data.

RNA Isolation and qPCR Analysis

Total RNA from LCM-procured cells was isolated using PicoPure® (Arcturus/Life Technologies) kits, which are optimized for extracting RNA from cells procured by LCM (23, 34). RNA in intact tissue sections, extracted with RNeasy® (Qiagen, Valencia, CA) kits, was analyzed prior to proceeding with LCM by utilization of the Bioanalyzer™ instrument and reagents (Agilent Technologies, Palo Alto, CA), which estimates integrity of total RNA through analysis of the 18S and 28S rRNA profiles given by electrophoretic separation, and a RIN (RNA Integrity Number) which provides a numerical estimate of RNA integrity of the sample. Total RNA from either intact tissue sections or LCM-procured cells was reverse transcribed, and cDNA obtained was diluted 10-fold in 2 ng/μl polyinositol (Sigma) and used for the qPCR reactions (23, 34). qPCR reactions were performed using a total volume of 10 μl, containing Power

Sybr® Green PCR Master Mix (Applied Biosystems), forward/reverse primers and cDNA obtained from the reverse transcription reaction. Primers were designed using Primer Express® 3.0 (Applied Biosystems). Relative gene expression analyses were performed using the $\Delta\Delta C_t$ method using β -actin (ACTB) as a reference gene. Expression of genes was compared to those present in Universal Human Reference RNA (Stratagene, La Jolla, CA) in order to obtain a relative expression level of target gene produced.

Statistical Analyses

T-tests and analysis of variance (ANOVA) were performed either in Microsoft® Excel or GraphPad Prism® Version 5 (GraphPad Software, La Jolla, CA). Box and whisker plots and Kaplan-Meier survival curves were generated in GraphPad Prism® Version 5. Univariate and multivariate cox regressions were performed with SPSS® Statistics 20 (SPSS Inc., Chicago, IL) for correlations with disease-free (DFS) and overall survival (OS). Survival calculations were performed using log2 transformations of relative gene expression data.

Results and Discussion

Relative Expression of Gene Candidates from Microarray Data

Expression profiling of cells using the microarray technique allows for high-throughput genetic analysis, with over 20,000 genes per sample analyzed in our investigation. Breast carcinomas consist of a variety of cell types and in order to obtain a high purity of cancer cells, laser-capture microdissection (LCM) was used to procure the carcinoma cells. Although non-microdissected ILC and IDC lesions have been genetically profiled in previous studies (35), LCM-procured cells offer the best opportunity for analyzing specific cell types without running the risk of including normal epithelial, adipose, or endothelial breast cells (9, 10).

Although there is evidence that tumors of the same histologic type have similar gene expression profiles from microarray regardless of their ER status (36), we first investigated both ER+ cancers and carcinomas regardless of their receptor status when performing t-tests ($p < 0.05$) to determine which genes were differentially expressed between Grade 1 IDC and ILC but similar between Grade 3 IDC and ILC. Initially, 15 gene candidates were identified, but 3 of the genes were expressed sequence tags (ESTs) and a commercially available probe could not be designed for those ESTs (Fig. 7). Also, the design of a probe for one of the gene candidates was unsuccessful, thus only 11 gene candidates were selected for further evaluation.

Due to the parameters employed for the initial gene selection, each was statistically differentially expressed ($p < 0.01$) among Grade 1 IDC and ILC and not differentially expressed ($p > 0.01$) between Grade 3 IDC and ILC (Fig. 7.) The 11 genes that were chosen for validation by quantitative polymerase chain reaction (qPCR) were BRWD1, CAPSL, CHRNA7, CMTM7, CRMP1, FAM210A, GSKIP, HBEGF, HYMAI, PAPPA, and LRBA. Box and Whisker Plots of the gene expression levels of the breast carcinoma cell types are shown in Figure 8 which provides a visual of their expression levels. A brief description of the 11-gene set is presented in Table 2.

Figure 8 continued onto next page.

Figure 8. Differences in gene expression levels of histologic subtypes using microarray analyses

Gene	UniGene ID	Description
BRWD1	Hs.654740	Bromodomain and WD repeat domain containing
CAPSL	Hs.55150	Calcyphosine-like protein
CHRNA7	Hs.511772	Cholinergic receptor, nicotinic, alpha 7 (neuronal)
CMTM7	Hs.440494	CKLF-like MARVEL transmembrane domain containing
CRMP1	Hs.135270	Collapsin response mediator protein
FAM210A	Hs.13034	Family with sequence similarity 210, member A
GSKIP	Hs.4104	GSK3B interacting protein
HBEGF	Hs.799	Heparin-binding EGF-like growth factor
HYMAI	Hs.657760	Hydatidiform mole associated and imprinted (non-protein coding)
PAPPA	Hs.643599	Pregnancy-associated plasma protein A, pappalysin
LRBA	Hs.480938	LPS-responsive vesicle trafficking, beach and anchor containing

Table 2. Gene set identified by microarray analyses and proposed for validation via qPCR.

Validation of Gene Candidates by qPCR Analyses

After identifying gene candidates for developing a clinically relevant molecular signature, it was necessary to validate the microarray results using qPCR analyses. Although the qPCR assay does not permit analyses of the vast number of genes as accomplished by the microarray technique, it is more accurate. This is due to the fact that microarray uses a single probe to amplify a gene, whereby qPCR uses both forward and backward probes that are necessary for amplification. However, intact frozen tissue sections were used for qPCR analyses in these studies rather than LCM-procured cells. Thus there is a slightly increased possibility that gene expression of non-cancerous breast cells were being analyzed. This possibility is greatly reduced due to the fact that we selected breast carcinoma specimens with elevated carcinoma cell content as described earlier. Also, instead of the 247 LCM-procured specimens analyzed in the microarray, the qPCR validation was performed using 58 whole tissue sections. Each of the specimens studied was from postmenopausal patients with ER+ carcinomas: n=19 for Grade 1 IDC; n=18 for Grade 3 IDC; n=21 for ILC.

Following validation of the 11 gene candidates by qPCR (Table 2, Table 3, Fig. 9), two genes were found to be differentially expressed to a degree of statistical significance ($p < 0.05$) (Table 3). The levels of the gene CRMP1, which is the collapsin response mediator protein 1, were overexpressed compared to that of the housekeeping gene, β -actin in ILC and but not in Grade 1 IDC (Fig. 9). From a previous report, CRMP1 appears to be involved in the suppression of cancer invasion and metastasis in lung cancer cells (37). The levels of the second gene HBEGF, which codes for the heparin-binding EGF-like growth factor, appear to be underexpressed compared that of β -actin in Grade 1 IDC and slightly overexpressed compared to the reference gene in ILC (Fig. 9). Overexpression of HBEGF has been found to promote breast metastasis and invasion specifically in ER- tumors (38), and to play a role in tumor aggressiveness in triple negative breast cancer (39).

Gene	Microarray Data			qPCR Data		
	mean Grade 1 IDC	mean ILC	P value	mean Grade 1 IDC	mean ILC	P value
BRWD1	0.425	-0.309	0.005	1.001	1.261	0.607
CAPSL	2.023	0.202	0.010	1.852	1.406	0.432
CHRNA7	-0.755	0.442	0.002	-1.234	-0.670	0.255
CMTM7	-1.443	0.212	0.007	-0.502	0.204	0.140
CRMP1	-0.331	1.074	0.009	-0.078	0.733	0.022
FAM210A	0.224	-0.328	0.005	-0.976	-1.030	0.787
GSKIP	0.378	-0.199	0.011	0.669	1.009	0.233
HBEGF	-0.376	0.512	0.004	-0.533	0.059	0.036
HYMAI	-0.840	0.281	0.008	-2.082	-1.583	0.524
PAPPA	-0.200	0.431	0.008	-1.579	-1.138	0.159
LRBA	0.533	-0.237	0.004	1.689	2.096	0.375

Table 3. T-test of microarray data and qPCR data comparing gene candidate expression between grade 1 IDC and ILC.

Figure 9 continued onto next page.

Figure 9. Validating differences in gene expression levels of histologic subtypes using qPCR

Pearson correlations were performed on the samples with which we had both qPCR and microarray data (n=29) to determine if the results of the two techniques correlated (Table 4). Only 4 of the 11 gene candidates (CAPSL, CMTM7, HBEGF, HYMAI) showed a statistically significant correlation ($p < 0.05$) between the assay platforms, which is not unexpected considering the vastly different protocol formats.

Pearson Correlation (n=29)		
Gene	Pearson r	P value
BRWD1	0.1329	0.4918
CAPSL	0.5903	0.0008
CHRNA7	0.3615	0.054
CMTM7	0.7963	< 0.0001
CRMP1	0.2915	0.1249
FAM210A	0.05411	0.7804
GSKIP	0.1628	0.3987
HBEGF	0.6981	< 0.0001
HYMAI	0.4594	0.0122
PAPPA - AJ420467	0.08267	0.6698
PAPPA - AI271743	0.1512	0.4337
LRBA	-0.04324	0.8271

Table 4. Pearson Correlation comparing expression results from microarray and qPCR of gene candidate.

Our preliminary results indicate that HBEGF and CRMP1 are differentially expressed in breast cancer subtypes. Interestingly, HBEGF was expressed to a significantly different degree between Grade 1 IDC and ILC using results from either microarray or qPCR (Fig. 8 & 9). Also expression levels appeared to be correlated between the two techniques. These initial results identify HBEGF expression as a potential marker differentiating between Grade 1 IDC and ILC. HBEGF, a ligand for EGFR and other ErbB receptors, has been implicated in enhancing the malignant potential of a number of cancers, including breast cancer (38). Although the results from qPCR analyses also suggest CRMP1 expression as a potential marker for distinguishing breast cancer subtypes, additional carcinoma specimens are required to validate these findings.

As described earlier, we observed that there was a noticeable difference in both disease-free and overall survival of breast carcinoma patients in our study population when one compares those exhibiting low grade (Grade 1) compared to high grade (Grade 3) carcinomas (Fig. 2). The risk of recurrence and survival are very different among the various grades which is extremely important considering that grading is known to be an independent predictor of survival and recurrence in invasive carcinomas (8). We also observed that there was a difference in DFS and OS of patients with Grade 1 IDC compared those with ILC (Fig. 2). Our preliminary findings clearly indicate that expression of HBEGF and CRMP1 in tissue biopsies may aid the pathologist in distinguishing the two histologic subtypes, thus serving as predictive biomarkers of breast carcinoma behavior.

IV. SUMMARY AND CONCLUSION

There are instances in which conventional pathologic tests to classify IDC and ILC are conflicting, and it is imperative to properly distinguish between these two types due to variable responses to endocrine treatments as well as differing prognoses. Conventional biomarkers such as ER, PR, and HER-2/neu offer little assistance in differentiation, in regard to both protein receptor status and gene expression. However, microgenomics may be of use in differentiating between the two most common types of breast cancer in women. Microarray analyses of LCM-procured cells have allowed us to examine the genomic profiles of specific tumor cells from 247 patients, and we have identified 11 genes candidate that could act as clinically relevant gene signatures. Gene expression levels retrieved from qPCR can be used to validate the candidate genes that were identified by microarray assays.

Two genes, CRMP1 and HBEGF, were identified as gene candidates and validated by qPCR as significantly differentially expressed between Grade 1 IDC and ILC. These two genes therefore merit further investigation as potential clinically relevant gene signatures that would discriminate between invasive lobular and ductal breast carcinomas. Future plans are to expand the patient sample and collect more data from qPCR so that more genes may be validated as statistically significant gene signatures to be used for clinical applications.

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