Differences in Gene and Protein Expression and the Effects of Race/Ethnicity on Breast Cancer Subtypes

Mariana Chavez-MacGregor1, Shuying Liu1, Debora De Melo-Gagliato1, Huiqin Chen1, Kim-Anh Do2, Lajos Pusztai6, W. Fraser Symmans3, Lakshmy Nair1, Gabriel N. Hortobagyi1, Gordon B. Mills4, Funda Meric-Bernstam5, and Ana M. Gonzalez-Angulo1,4

Abstract

Background: Differences in gene or protein expression patterns between breast cancers according to race/ethnicity and cancer subtype.

Methods: Transcriptional profiling was performed using Affymetrix HG-U133A platform in 376 patients and reverse phase protein array analysis (RPPA) was done for 177 proteins in 255 patients from a separate cohort. Unsupervised clustering was conducted, as well as supervised comparison by race and tumor subtype. Standard statistical methods, BRB-Array tools, and Ingenuity Pathways software packages were used to analyze the data.

Results: Median age was 50 years in both the cohorts. In the RPPA cohort, 54.5% of the tumors were hormone receptor–positive (HR-positive), 20.7% HER2-positive, and 24.7% triple-negative (TNBC). One hundred and forty-seven (57.6%), 47 (18.43%), and 46 (18.1%) of the patients were White, Hispanic, and Black, respectively. Unsupervised hierarchical clustering of the protein expression data showed no distinct clusters by race (P values were 0.492, 0.489, and 0.494 for the HR-positive, HER2-positive, and TNBC tumors respectively). In the gene expression cohort, 54.2% of the tumors were HR-positive, 16.5% HER2-positive, and 29.3% TNBC. Two hundred and sixteen (57.5%), 111 (29.52%), and 32 (8.52%) patients were White, Hispanic, and Black, respectively. No probe set with a false discovery rate (FDR) of <0.05 showed an association with race by breast cancer subtype; similar results were obtained using pathway and gene set enrichment analysis methods.

Conclusions: We did not detect a significant variation in RNA or protein expression comparing different race/ethnicity groups of women with breast cancer.

Impact: More research on the complex network of factors that result in outcomes differences among race/ethnicities is needed.

Introduction

Breast cancer is the most common cancer among women of every major ethnic group in the United States. It is estimated that there are nearly 3 million women living in this country with a history of invasive breast cancer, and an additional 226,870 women were diagnosed in 2012 (1).

Although the incidence rate of breast cancer is lower in Black and Hispanic women (2) compared with White women, there are some serious disparities in breast cancer–related death rates among different ethnic groups. According to recent data, when evaluating outcome independently of stage at diagnosis, Black women with breast cancer have worse outcomes, and Hispanics, on the other hand, experience slightly improved outcomes compared with their White counterparts. (3–5). Survival disparities found between those groups are poorly understood and probably stem from many causes.

Socioeconomic factors have been proposed to account for the poorer survival outcomes seen in those groups. It has been well described that Black and Hispanic women tend to be diagnosed with more advanced clinical stages (6–10). Lantz and colleagues have shown that even after control for study site, age, and individual socioeconomic factors, the odds of early detection were still significantly less for Hispanic and Black women when compared with White women (9).

Lower socioeconomic status among Black women has been assigned as one of the potential causes for the poor outcomes in this patient population (11). Nevertheless, some authors demonstrated that the race itself is a significant and independent predictor of poor outcomes...
from breast cancer, even after accounting for socioeconomic status (12). Biologic tumor differences among Black/Hispanics and White women are also a matter of debate in the literature. Previous studies have demonstrated some differences in the distribution of breast cancer subtypes among Black women. It has been consistently demonstrated that among Black women, the rates of a more aggressive breast cancer subtype, the so-called triple-negative breast cancer (TNBC), are higher, and a similar pattern has been described among Hispanics (13–15). This subtype of breast cancer correlates with a genetic signature of basal-like tumors, which is associated with aggressive histologic features, BRCA mutation carriers, and poor prognosis (14, 15). Although early studies of BRCA mutations suggested that they were relatively rare among Black women (16), many studies have found BRCA1 and more frequently BRCA2 mutations in this patient population (17, 18). Other epidemiologic factors in addition to differences in the tumor microenvironment and distinct genetic features have also been described in previous studies (19–21).

The factors contributing to the discrepancies in survival outcomes between Black, Hispanics, and White patients are complex and could relate to both social and biologic differences. It is thus of extreme importance to determine whether genetic differences play a role in this scenario, as this could change the approach to decreasing the discrepancies in outcomes. One approach to identify potential targets mediating biologic differences between races is to compare RNA and protein expression in breast cancer tissue from patients of a variety of ethnicities.

The purpose of this study was to evaluate whether there were differences in gene or protein expression patterns between primary invasive breast cancers according to race/ethnicity and breast cancer subtype.

Materials and Methods

Pretreatment fine-needle aspirations (FNA) specimens were collected during a prospective biomarker discovery study (LAB99-0402) and a prospective neoadjuvant clinical trial (LAB99-0325) at the University of Texas MD Anderson Cancer Center from patients with early-stage breast cancer who subsequently received neoadjuvant chemotherapy. Race was self-reported as White, Black, Hispanic, Asian, or other. Tumor subtypes were divided as triple-negative (TNBC), hormone receptor–positive/HER2-negative (HR-positive), and HER2-positive/with any (estrogen receptor) ER status (HER2-positive). The laboratory study was approved by the Institutional Review Board and all patients signed informed consent.

Two hundred and fifty-five FNA samples for functional proteomics analysis were snap-frozen and stored at −80°C. Reverse phase protein array analysis (RPPA) was performed. Briefly, protein lysates were obtained by sonication or homogenization of tumor tissue with lysis buffer, manually diluting in 5-fold serial dilutions, normalized to 1 μg/μL concentration and printed onto nitrocellulose-coated glass slides (FAST Slides; Schleicher & Schuell BioScience, Inc.) with an automated Aushon Arrayer (Aushon Biosystems). Slides were probed with 177 validated primary antibodies (Supplementary Table S1) and signal was amplified using a 3,3’-diaminobenzidine tetrachloride (DAB)–based Dako Signal Amplification System (Dako). A biotinylated secondary antibody was used as a starting point for signal amplification. Signal intensity was measured by scanning the slides and quantified with MicroVigene software (VigeneTech Inc.) to generate sigmoidal signal intensity concentration curves for each sample. Antibodies were selected on the basis of markers currently used for breast cancer classification due to their value in treatment decisions, targets implicated in breast cancer signaling, and targets implicated in the signaling of the other cancer lineages, as well as the availability of high quality that passed a strict validation process.

Three hundred seventy-six FNA samples for gene expression analysis were collected into RNA and later stored at −80°C. Gene expression profiling was performed with Affymetrix U133A gene chips as previously described (22, 23). Complete gene expression data are available in the Gene Expression Omnibus (GEO) under accession numbers GSE16716 and GSE20271.

Statistical analysis

Patient characteristics were described and compared by race/ethnicity group with a χ² test, Fisher exact test, or Kruskal–Wallis rank sum test, as appropriate.

The RPPA spot signal intensity data from MicroVigene were processed using the R package SuperCurve (http://bioinformatics.mdanderson.org/OOMPA), and normalized by a median polish method. Gene expression data sets were normalized with MAS5 algorithm, mean centered to 600 and log₂ transformed before further analysis. We removed probe sets with average expression values less than the lowest 25% to reduce noise from low expressed probe sets.

As the first step of exploring how patients cluster by race, unsupervised hierarchical clustering (Pearson correlation, average linkage) across the RPPA data was conducted for each breast cancer subtype. An ANOVA test was performed to assess differential protein expression between races by each subtype, with false discovery rate (FDR) corrected by using the beta-uniform mixture (BUM) model. If the ANOVA tests indicated that there was a significant difference in the means among the races, we performed multiple comparisons between all the pair-wise means to determine the pairs that were different. The least square and the Kolmogorov–Smirnov test, from Gene Set Expression Comparison module of BRB-ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html), were used to compare proteomics data by using all 177 protein biomarkers simultaneously as inputs among the race/ethnicities by breast cancer subtypes.

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Analogous statistical methods were performed to analyze the transcriptional data. We validated the RNA signature for differential races identified patients with HER2-positive tumors using Linear Discriminant Analysis (LDA) on an independent test set of 59 HER2-positive samples. Finally, we also conducted pathway analyses comparing races/ethnicities within each subtype, respectively, using Ingenuity Pathway Analysis (IPA) software (http://www.ingenuity.com).

The software R 2.11.2 (R Development Core Team 2010) was used to analyze the data. FDR less than 0.05 were considered significant.

Results

Functional proteomics

Table 1 summarizes the patient’s characteristics by race/ethnicity. There were 54.5% HR-positive, 20.7% HER2-positive and 24.7% TNBC among 147 (57.6%) White, 47 (18.4%) Hispanic, and 46 (18.1%) Black patients. Compared with other groups, Hispanic patients were younger ($P = 0.006$), and Black patients had more frequently TNBC, ($P = 0.0006$).

Heat maps from unsupervised clustering analysis by breast cancer subtype are presented in Fig. 1, showing no indication of an orderly pattern of races/ethnicities in the clustering. Furthermore, after multiplicity adjustment by applying a BUM model to the $P$ values from the ANOVA test of race/ethnicity by breast cancer subtype, none of the 177 proteins was considered differentially expressed (FDR < 0.05) among different races/ethnicities by breast cancer subtypes (histograms presented in Supplementary Fig. S1).

Table 2 summarizes the $P$ values least square and Kolmogorov–Smirnov permutation tests (from using BRB-Array Tools) showing no evidence of simultaneous protein expression differences between the different breast cancer subtypes according to race/ethnicity.

Transcriptional profiles

Table 3 summarizes the patient’s characteristics by race/ethnicity. There were 54.2% HR-positive, 16.5% HER2-positive, and 29.3% TNBC among 216 (57.8%) White, 111 (29.5%) Hispanic, and 32 (8.5%) Black patients. Compared with other groups, Hispanic patients presented with more advanced stages ($P < 0.0001$). No other differences were seen.

After excluding probes with low variance, 16,438 probe sets were included in the analysis. The BUM models of $P$ values from ANOVA tests of races/ethnicities by breast cancer subtype (histograms presented in Supplementary Fig. S2) showed that three probe sets from HR-positive disease, 384 probe sets from HER2-positive disease, and no probe sets from TNBC had FDR less than 0.05. Unsupervised clustering of the significant 384 probe sets with...
FDR < 0.05 among the 62 HER2-positive tumors showed differences according to race/ethnicity. However, validation in an independent set of 59 HER2-positive tumors across the significant 384 probes showed no pattern in the race comparisons (Supplementary Fig. S3).

IPA showed no obvious difference among races within breast cancer subtypes. The disease and disorder–related pathways of each subtype are bar plotted in Fig. 2.

Discussion

In summary, our results indicate that there are no large-scale gene and protein expression differences between breast cancers according to race/ethnicity and tumor subtype.

Previously, Grunuda and colleagues evaluated the expression of 84 genes involved in breast cancer prognosis, response to therapy, estrogen signaling, and tumor aggressiveness in age- and stage-matched paraffin-embedded breast cancer specimens form Black and White patients. They observed that twenty genes were differentially expressed between the two groups. Black women were significantly more likely to display aberrations in G1–S cell-cycle regulatory genes, decreased expression of cell-adhesion genes, and low to no expression estrogen pathway targets, which may confer a more aggressive behavior to the disease (20).

A previous study also suggested that there might be differences in the tumor microenvironment between African–American and European–American patients with breast cancer, with higher microvessel density and macrophage infiltration in African–American compared with the European White population (21).

Other tumor genetic differences previously found between Black and White women that could help understand the differences in outcome include more frequent genome copy-number gains and losses in Blacks (24), more prevalent expression of cyclin D1 (25), higher quantities of p16, p53, and cyclin E (14, 26, 27), differences in the methylation status for the CDH13 gene (tumor-suppressor gene; ref. 28), increased frequency of the CYP 3A4-G (mutated variant from Cytochrome P450 3A4; ref. 29), and others.

Because proteins are the immediate effectors of cellular behavior, interrogation of the functional proteome is likely to complement the data derived from transcriptional profiling. Thus, the integrated study of the expression and activation of multiple proteins and signaling pathways has the potential to provide powerful classifiers and predictors in breast cancer, as protein levels and function depend not only on translation but also on posttranslational modifications such as cleavage, degradation, phosphorylation, ubiquitination, and prenylation. Therefore, functional proteomic profiling

<table>
<thead>
<tr>
<th>Breast cancer subtype</th>
<th>LS permutation</th>
<th>P</th>
<th>KS permutation</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Hormone receptor-positive</td>
<td>0.49231</td>
<td>0.49231</td>
<td>0.55187</td>
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<tr>
<td>HER2-positive</td>
<td>0.48987</td>
<td>0.48987</td>
<td>0.6114</td>
<td>0.6114</td>
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<tr>
<td>Triple negative</td>
<td>0.494</td>
<td>0.494</td>
<td>0.65038</td>
<td>0.65038</td>
</tr>
</tbody>
</table>

Abbreviations: LS, least square; KS, Kolmogorov–Smirnov.
can provide more information about the pathways that are actually activated and involved in tumor proliferation and may theoretically yield more direct answers to functional and pharmacologic questions than transcriptional profiling alone (30). It is possible that, genetic alterations and protein expression can play an important role on the different outcomes identified between the Black Hispanics and White patients.

Many variables other than genetics and socioeconomic factors can play a role in determining the different outcomes between races. It has been well described that epidemiologic differences exist among patients with breast cancer (19, 21). We observed that Hispanic patients were younger at diagnosis, presented with more advanced stages, and were more likely to have HER2-positive tumors.

However, despite the epidemiologic characteristics, stage distribution and tumor subtype differences, when analyzing gene and protein expression, our results are consistent with others that have failed to demonstrate a biologic difference among patients with breast cancer according to race/ethnicity. Wang and colleagues (28) investigated the relationship between tumor DNA methylation status and patient characteristics in African–American and European–American women with breast cancer. Overall, they could not detect any methylation differences between African–American and European–American patients in ER-positive disease. The only difference found was in the young (less than 50 years of age) and ER-negative African–American patients, who had a significantly higher methylation index at the CDH13 locus than matched European–American patients. No other methylation differences were found between the two patient populations in the other five tumor-suppressor genes studied, corroborating the hypothesis that these tumors do not demonstrate marked biologic differences.

Finally, one cannot exclude that discrepancies in the treatment offered for Blacks and Hispanics had an impact on survival outcomes. African–American patients frequently experience underuse of appropriate adjuvant therapy in clinical practice (31), are less likely to complete therapy (32) and are more likely to experience a delay in the initiation of adjuvant chemotherapy (33, 34). Evaluating outcome was out of the scope of this study; however, all the patients included were treated at our institution in a standard manner or as part of a clinical trial. Therefore, it is very unlikely that the patients received suboptimal or differential therapies.

To fully appreciate our findings, several strengths and limitations should be mentioned. To our knowledge, this is the first study that compared differences among racial/ethnic groups in a proteomic level. Other studies that previously evaluated the breast cancer genetic patterns between races did not use the same methodology. Several studies have shown that RPPA technology is effective in mapping intracellular signaling networks in cell lines (28,

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total N (%)</th>
<th>Black N (%)</th>
<th>Hispanic N (%)</th>
<th>White N (%)</th>
<th>Other N (%)</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Age at diagnosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Minimum</td>
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<td>32</td>
<td>29</td>
<td>26</td>
<td>38</td>
<td>0.0722</td>
</tr>
<tr>
<td>Median</td>
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<td>49</td>
<td>48</td>
<td>51</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
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<td>75</td>
<td>77</td>
<td>80</td>
<td>69</td>
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<tr>
<td>Hormone receptor-positive</td>
<td>204 (54.26%)</td>
<td>13 (40.63%)</td>
<td>58 (52.25%)</td>
<td>122 (56.04%)</td>
<td>11 (64.71%)</td>
<td></td>
</tr>
<tr>
<td>HER2-positive</td>
<td>62 (16.49%)</td>
<td>4 (12.50%)</td>
<td>17 (15.32%)</td>
<td>36 (16.67%)</td>
<td>5 (29.41%)</td>
<td></td>
</tr>
<tr>
<td>Triple negative</td>
<td>110 (29.26%)</td>
<td>15 (46.88%)</td>
<td>36 (32.43%)</td>
<td>58 (26.85%)</td>
<td>1 (5.88%)</td>
<td>0.0712</td>
</tr>
<tr>
<td>Menopausal status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre/perimenopausal</td>
<td>174 (46.28%)</td>
<td>13 (40.63%)</td>
<td>62 (55.86%)</td>
<td>90 (41.67%)</td>
<td>9 (52.94%)</td>
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<tr>
<td>Postmenopausal</td>
<td>198 (52.66%)</td>
<td>19 (59.38%)</td>
<td>46 (41.44%)</td>
<td>125 (57.87%)</td>
<td>8 (47.06%)</td>
<td>0.0518</td>
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<td>Histology</td>
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<tr>
<td>Ductal</td>
<td>360 (95.74%)</td>
<td>31 (96.88%)</td>
<td>108 (97.30%)</td>
<td>205 (94.9%)</td>
<td>16 (94.12%)</td>
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<tr>
<td>Other</td>
<td>16 (4.26%)</td>
<td>1 (3.13%)</td>
<td>2 (2.70%)</td>
<td>11 (5.09%)</td>
<td>1 (5.88%)</td>
<td>0.6388</td>
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<td>Nuclear grade</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>24 (6.38%)</td>
<td>2 (6.25%)</td>
<td>2 (1.80%)</td>
<td>19 (8.80%)</td>
<td>1 (5.88%)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>141 (37.50%)</td>
<td>8 (25.00%)</td>
<td>33 (29.73%)</td>
<td>94 (43.52%)</td>
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</tr>
<tr>
<td>3</td>
<td>180 (47.87%)</td>
<td>22 (68.75%)</td>
<td>46 (41.44%)</td>
<td>102 (47.22%)</td>
<td>10 (58.82%)</td>
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<td>Stage</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>I</td>
<td>7 (1.86%)</td>
<td>0 (0.00%)</td>
<td>3 (2.70%)</td>
<td>4</td>
<td>1.85 (0.00%)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>192 (51.06%)</td>
<td>13 (40.63%)</td>
<td>30 (27.03%)</td>
<td>137 (63.43%)</td>
<td>12 (70.59%)</td>
<td></td>
</tr>
<tr>
<td>Ill and IV</td>
<td>176 (46.81%)</td>
<td>19 (59.38%)</td>
<td>78 (70.27%)</td>
<td>74 (34.26%)</td>
<td>5 (29.41%)</td>
<td>&lt;0.0001</td>
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</tbody>
</table>
35–38) and the use of this methodology is an advantage to our study.

We have included a large patient population that contains an important proportion of minority patients with breast cancer. Even large gene analyses like The Cancer Genome Atlas (TCGA) did not have a good representation of minorities (39). By evaluating potential differences according to race/ethnicity according to the tumor subtype, the number of patients in each category was relatively small and we were not able to perform subgroup analyses. In addition, the number of tumors analyzed does not allow us to confidently exclude small-scale expression differences in a limited number of proteins or RNAs. In our study, we did not examine the potential differential distribution of DNA nucleic acid polymorphisms, somatic mutations, gene rearrangements, or copy-number aberrations by race/ethnicity, which could account for biologic differences between ethnicities.

Our study is prone to the challenges associated with analysis according to race/ethnicity. We acknowledge that particularly Hispanics and Blacks represent heterogeneous populations where a mix of different ancestries is likely to exist. We were not able to obtain ancestry data and did not perform ancestry gene analysis. In addition, race/ethnicity was self-reported and we cannot rule out the presence of misclassification. Nevertheless, most of
the studies that evaluated the outcomes between the different ethnicities used the same classification system as in our study.

In conclusion, based upon the data in this study, we were not able to detect significant variation in RNA or protein expression comparing different ethnicity groups of women with breast cancer.

Further studies, with larger sample sizes and deeper molecular analyses like TCGA (39), are needed. In addition, this type of studies must include a diverse patient population, so they can provide a better understanding about the complex network of factors that ultimately might result in differential survival outcomes among patients with breast cancer.

Disclosure of Potential Conflicts of Interest
G.B. Mills received commercial research grants from AstraZeneca, Han AllBio Korea, and GlaxoSmithKline; has ownership interest (including patents) in Calena Pharmaceuticals, PTW Ventures, and Spindle Top Ventures; and is a consultant/advisory board member of AstraZeneca, Bind, Critical Outcome Technologies, Han AllBio Korea, Nuevement, and Symphogen. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: M. Chavez-MacGregor, D. De Melo-Gagliato, G.N. Hortobagyi, A.M. Gonzalez-Angulo

References

Development of methodology: S. Liu, D. De Melo-Gagliato, L. Pusztai, G.B. Mills, A.M. Gonzalez-Angulo

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Liu, D. De Melo-Gagliato, L. Pusztai, W.F. Symmans, A.M. Gonzalez-Angulo

Analysis and interpretation of data (e.g., statistical analysis, biostatis-
tics, computational analysis): M. Chavez-MacGregor, S. Liu, D. De Melo-

Writing, review, and/or revision of the manuscript: M. Chavez-MacGregor, D. De Melo-Gagliato, K.-A. Do, L. Pusztai, W.F. Symmans, G.N. Hortobagyi, G.B. Mills, F. Meric-Bernstam, A.M. Gonzalez-Angulo

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G.B. Mills, A.M. Gonzalez-Angulo

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