**Abstract.** Background: African-American women develop more aggressive breast cancers and at an earlier age compared with Caucasian women. Materials and Methods: We compared gene expression profiles of breast cancer cell lines that were developed from African-American and Caucasian patients to identify biological differences in breast cancers that develop in these groups. Real-time PCR was used to evaluate mRNA expression in cell lines and in a series of breast cancer cases. Gene microarray signal intensities were also analyzed in the International Genomics Consortium Expression Project for Oncology (expO) dataset. Results: 17'-Hydroxysteroid dehydrogenase type 2 (17HSD 2) gene and mRNA expression were significantly higher in the African-American cell lines (p<0.05). However, 17HSD 2 expression did not differ significantly between the two cohorts in either our clinical series or the expO dataset. 17HSD 2 expression was found to be predictive of younger age at diagnosis and estrogen receptor status. Conclusion: Overexpression of 17HSD 2 in African-American breast cancer may contribute to the increased proportion of estrogen receptor-negative breast cancers and worse clinical outcome among African-American patients.

Breast cancer is the most common cancer in US women, and the second leading cause of cancer-related mortality in women. Clinical advances since the 1970s have helped reduce breast cancer mortality rates; however, this reduction has been seen mainly among Caucasian (Cau) patients creating a divergence in mortality rates between African-American (AA) and Cau patients with this disease (1). Despite an estimated 13% lower incidence of breast cancer among AA women compared with Cau women, breast cancer mortality rates remain 28% higher among AA women (2). Several studies now suggest that beyond socioeconomic factors, differences in tumor biology may contribute to this difference in outcome (3). In addition to being diagnosed at a later disease stage, breast cancers that develop in AA women occur at a younger age and are more often of higher grade and less often estrogen receptor (ER) positive, compared with those that develop in Cau women (4). Little is known about potential racial and ethnic differences in the molecular mechanisms that may lead to these differences in tumor biology.

Several studies have attempted to examine genetic factors that may underlie the more aggressive behavior of breast cancer in AA women. Previous reports of AA breast cancer have uncovered unique mutations in the brca1 and brca2 breast cancer susceptibility genes (5-8), both distinct and more frequent p53 gene alterations (9, 10), and polymorphisms in the ras gene (11). In a case-control study of breast cancer in AA women, Porter et al. demonstrated that, compared to Cau controls, tumors in AA women have a higher mitotic index; overexpression of cyclin E, p16, and p53; and low expression of cyclin D1 at diagnosis (12). We previously found that expression rates of cyclin D1, p53, p27, and p21 were similar in 200 cases of Cau and AA breast cancer that were matched on ER status and other clinicopathological variables, but that cyclin D1 overexpression was significantly associated with ER status in only the Cau and not the AA cases (13). Further evaluations of possible clinical targets have revealed no significant differences in the level of expression of human epidermal growth factor receptor (HER)-2, Ki-67, epidermal growth factor receptor (EGFR), carcinoembryonic antigen (CEA), cathepsin D, or pS2 between these two groups (10, 14-16).

Gene expression studies using DNA microarrays have identified several major breast cancer subtypes with markedly different prognoses (17, 18) and differences in prevalence among AA and Cau women (19). The subtypes
include two main groups of ER-negative tumors, basal-like ("triple negative") and HER-2 positive/ER-negative, and two types of ER-positive tumors, luminal A and luminal B. These subtypes can be distinguished by use of immunohistochemical surrogates. The basal-like subtype typically shows low expression of HER-2 and ER and high expression of genes characteristic of the basal epithelial cell layer and carries a worse prognosis than the luminal subtypes. In the Carolina Breast Cancer Study, a population-based, case-control study that oversampled premenopausal and AA women, Carey et al. found that the basal-like subtype was more prevalent among premenopausal AA women compared with postmenopausal AA women and non-AA women of any age, whereas the luminal A subtype was less prevalent among AA women (19). Compared with the luminal A subtype, the basal-like tumors had significantly more TP53 mutations, higher mitotic index, more marked nuclear pleomorphism, higher combined grade, and shorter survival. This large population study thus suggests how differing tumor biology may contribute to the worse prognosis for AA breast cancer patients.

While microarrays have been used to characterize the biologic subtypes of breast cancer, no study has yet directly compared the gene expression profiles of breast cancers that develop in AA and Cau patients. In this study, we performed a microarray analysis to try to identify genetic changes between AA and Cau breast tumors that may contribute to their differing prognosis and may represent new clinical targets for the treatment and prevention of breast cancer in AA women. We evaluated the expression of over 22,000 genes in six breast cancer cell lines derived from AA and Cau patients and discovered that the gene 17β-hydroxysteroid dehydrogenase type 2 (17HSD 2), which plays a key role in estrogen metabolism, is significantly overexpressed in those cell lines developed from AA patients. Our study suggests a novel biological difference in AA breast cancer that may contribute to the more aggressive phenotype of breast cancer seen in these patients.

Materials and Methods

Cell lines. Six commercially available human breast cancer cell lines (American Type Culture Collection, Manassas, VA, USA) were studied. Three were developed from AA patients (ZR75-30, MDA-MB-175, MDA-MB-468), and three from Cau patients (MCF7, MDA-MB-231, MDA-MB-436) (20, 21). MDA-MB-231 and MDA-MB-468 were grown in MEM supplemented with 10% fetal bovine serum (FBS). MDA-MB-175 was grown in Leibovitz’s Medium L-15 with 10% FBS, and MDA-MB-436 was grown in Leibovitz’s Medium L-15 supplemented with 10% FBS, insulin (10 mcg/mL), and glutathione (16 mcg/mL). MCF7 was grown in DMEM with 10% FBS. ZR75-30 was grown in RPMI with 10% FBS. All cell culture media and FBS were obtained from Life Technologies (Grand Island, NY, USA). All of the cell lines were maintained at 37°C in a 5% CO2 atmosphere. Cells were plated in 10 cm culture dishes at concentrations to yield 60-70% confluence within 24 hours. Cells were harvested for RNA isolation 24 hours after plating.

Tissue samples. Human breast cancer tissue samples were obtained from the Herbert Irving Comprehensive Cancer Center (HICCC) Tumor Bank at Columbia University, NY, USA. Clinical specimens were obtained at surgery between 1994 and 2003 and were immediately for further analyses, including RNA extraction. Patient identifiers, including name and hospital medical record number, were not used in order to ensure patient confidentiality. This research tumor bank was approved by the Columbia University Medical Center Institutional Review Board (IRB) prior to specimen acquisition (IRB #X0898). Self-reported and physician-reported classifications of race were used to identify cases. Clinicopathological data available for corresponding banked paraffin-embedded tissue specimens included: patient age at diagnosis, tumor grade, tumor size, tumor stage, HER-2 expression (determined using the DAKO system scale for membrane staining or quantitative immunohistochemistry using the CAS200 Quantitative Image Analyzer (Becton Dickinson, San Jose, CA, USA) and Quantitative Oncogene product program; positive – 2+ or 3+ membrane staining by the DAKO scale or greater than 0.1 pg/cell, negative – 0 or 1+ membrane staining by the DAKO scale or less than 0.1 pg/cell), ER and PgR status (positive or negative), S-phase fraction, MIB-1, DNA ploidy, recurrence status, and survival.

Microarray analysis. cRNA preparation and array hybridization were conducted in collaboration with the Columbia University Microarray Project (CUMAP). Total cell RNA was isolated using trizol reagent (Invitrogen, Carlsbad, CA, USA) and cleaned and precipitated with the Qiagen RNeasy Cleanup Kit (Qiagen). Slides were washed and scanned using a confocal laser scanner to generate fluorescence intensities.

Signal intensities were normalized using (robust multichip analysis) RMA and then uploaded into the GeneTraffic program (Stratagene, La Jolla, CA, USA). Within this program, signal intensities were filtered to limit data to genes that had at least one observation with a two-fold change in intensity from baseline. A t-test comparison with Benjamini-Hochberg correction for multiple analyses was used to identify genes expressed differently between the two groups.

Real-time PCR. Real-time PCR experiments were conducted in an ABI Prism 7700 sequence detector (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). All reactions were semi-quantitative and evaluated the relative expression of 17HSD 2 mRNA calibrated to the expression of 17HSD 2 in the cell line ZR75-30. All experiments were performed in duplicate. RNA was isolated from cell lines and frozen tissues using Trizol (Invitrogen) and then purified with the Qiagen RNeasy Cleanup Kit (Qiagen). During purification, RNA was further treated with DNase1
RNA integrity was confirmed by agarose gel electrophoresis and the concentration was determined by measuring OD260nm in the BioPhotometer (Eppendorf, New York, NY, USA). cDNA was generated using Superscript II (Invitrogen) with random hexamer primers. 17HSD 2 primers were designed according to the report of Kasai et al. (22), and sequences were sense 5'-CTGAGGAA TTGGCAAGAAC-3' and antisense 5'-AAGAAGCTCCCCCAT CAGTTG-3'. β-actin served as an endogenous control for the real-time PCR. β-actin primer sequences were sense 5'-CCAGGC ACCAGGGCGTGATG-3' and antisense 5'-CGGCCAGCCAGGT CCGAGCG-3' (23). All primers were synthesized by Invitrogen and quality confirmed in a PCR that demonstrated the absence of primer dimers, a single band on agarose gel electrophoresis, and only one peak in dissociation curves. All PCR products were in the 140-180 bp range.

For the real-time PCR experiments, each reaction consisted of 5 μL of cDNA (approximately 10 ng input RNA) added to 45 μL of SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA). This master mix contained 10x SYBR buffer (5 μL), 25 mM MgCl2 (6 μL), 10 mM dNTP (4 μL), amplitaq gold (0.25 μL), dungs amperase (0.5 μL), 20 μM sense primer (1.5 μL), 20 μM antisense primer (1.5 μL), and DEPC water (26.25 μL). Cycling conditions were as follows: 50°C for 2 minutes, 95°C for 2 minutes, and then 50 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. No template control and no reverse transcription control were included in each assay and all samples were run in duplicate. The ABI Prism 7700 determined the threshold cycle (Ct) for each sample. C is inversely proportional to starting cDNA concentration. Relative 17HSD 2 expression was determined by calculating the ratio of 17HSD 2 input to β-actin input for each sample from standard curves for 17HSD 2 and β-actin generated during the reaction. 17HSD 2 expression values were then normalized to the expression level of 17HSD 2 in the cell line ZR75-30.

Data mining. To evaluate 17HSD 2 expression in a larger clinical sample set, we searched the National Library of Medicine Gene Expression Omnibus Database to identify breast cancer datasets that used the Affymetrix platform U133. Using the search terms "African" and "breast," the International Genomics Consortium Expression Project for Oncology (expO) was identified. The expO initiative has procured frozen tissue samples with corresponding paraffin block sections and clinical information for patients with a variety of different tumor types. Using microarray technology, gene expression has been assayed on these tumor specimens and is available publicly online (http://www.intgen.org). Examination of this dataset revealed 227 breast cancer samples collected from Cau and AA patients (212 Cau, 15 AA). 17HSD 2 and 17HSD 1 expression levels, age, and where available, ER status were recorded from this dataset and used to investigate associations between 17HSD 2 expression and race, age, or ER status.

Statistical analyses. The number of tissue specimens evaluated was limited by the number of available AA patient samples in our tumor bank. Thus, formal sample size and power calculations were not performed. For the cell line data, a linear mixed model was used to assess the difference in 17HSD 2 expression (log scale) by pooling expression data from all real-time PCR experiments. For the tissue samples, linear mixed models were used to assess associations between 17HSD 2 expression (log scale, dependent variable) and demographic and clinical parameters such as race and ER status. A random patient effect was added in the model to account for correlations among data from the same patient. Due to the limited sample size, only one factor (demographic or clinical parameter) was included in the model at a time. Mean ratio for the effect on each dependent variable was calculated. For discrete factors, the mean ratio was the mean ratio of 17HSD 2 expression with respect to a base level of the variable. For continuous covariates, the mean ratio was the mean ratio of 17HSD 2 expression with respect to every unit increase in the variable. Wald's test was used to draw statistical inference. For the expO dataset, linear models were used to assess associations between 17HSD 2 expression (log scale, dependent variable) and race, age, and ER status. Significant factors were identified by forward stepwise model selection: factors that were significant (at the 0.05 level) in the univariate models were included in a final model. The interaction effects of race and ER status were also evaluated. Statistical significance was defined as p<0.05.

Results

Expression of 17HSD 2 in breast cancer cell lines. Six breast cancer cell lines, three developed from AA patients (ZR75-30, MDA-MB-175, MDA-MB-468) and three from Cau patients (MCF7, MDA-MB-231, MDA-MB-436), were used in a microarray experiment that evaluated the expression by each cell line of 22,283 genes present in the Affymetrix GeneChip Human Genome U133. Signal intensities for gene expression in each cell line were normalized using RMA. To filter out arrays of poor quality, we limited data to genes that had at least one observation with a two-fold change in intensity from baseline. A total of 3899 genes satisfied this filtering criterion. A t-test comparison with Benjamini-Hochberg correction identified two genes that were expressed differently between the two groups (AA versus Cau) at the p<0.05 level: 17HSD 2 and transcription factor AP2α (Table 1 and data not shown). Table 1 lists the signal intensities for 17HSD 2 for each cell line. For the Cau cell lines, MDA-MB-231, MDA-MB-436, and MCF7, the signal level for 17HSD 2 was 5.25, 5.52, 5.38, respectively. In contrast, for the AA cell lines, MDA-MB-175, MDA-MB-468, and ZR75-30, the signal level was 9.36, 8.93, and 8.97, respectively. At the p<0.05 level, expression of 17HSD 2 was found to be significantly higher among the AA cell lines compared to the Cau ones. There was a nearly two-fold greater expression of 17HSD 2 among the AA cell lines compared to the Cau ones. There was no significant difference in 17HSD 1 signal intensities between AA and Cau cell lines (data not shown). The ratio of 17HSD 2/17HSD 1 varied significantly between the two groups due to the difference in 17HSD 2 expression (p=0.01). Of note, there was no significant difference in 17HSD 2 signal levels based on ER expression (positive versus negative; data not shown).

Relative expression of 17HSD 2 mRNA in breast cancer cell lines. We performed a real-time PCR experiment to
quantify the relative level of 17HSD 2 mRNA expression in each cell line in order to confirm the results of the microarray analysis. The level of 17HSD 2 expression was calculated relative to the level of β-actin expression and was normalized to 17HSD 2 expression in the cell line ZR75-30. Figure 1 shows the relative level of 17HSD 2 expression in each cell line. The relative level of expression of 17HSD 2 in the AA cell lines ranged from 1 to 45. In the Cau cell lines, the level ranged from 5x10⁻⁵ to 0.2 and all were at least an order of magnitude less than the expression level in the AA samples. At the mRNA level, the expression of 17HSD 2 was significantly higher in the AA breast cancer cell lines compared to the Cau cell lines (p=0.047), confirming the results of the microarray analysis.

Relative expression of 17HSD 2 mRNA in breast cancer specimens. We evaluated the expression of 17HSD 2 in a cohort of 16 breast cancer tissue specimens obtained from the HICCC Tumor Bank at Columbia University. Using real-time PCR, we determined the relative 17HSD 2 mRNA expression levels (Figure 2). Figure 2 also indicates the race of the patient from whom the tissue was obtained. In this small series, the level of 17HSD 2 expression did not vary as consistently by race as in the cell lines. The average expression of 17HSD 2 among the samples from AA patients was 2.66, and among those from Cau patients was 0.32 (p=0.57).

Association of 17HSD 2 mRNA expression with clinical parameters. 17HSD 2 expression levels in the 16 tissue specimens were correlated with key clinicopathologic parameters. Table II indicates the mean ratio and p value for each parameter. Age at diagnosis varied significantly with 17HSD 2 expression, and a younger age at diagnosis was associated with higher relative levels of 17HSD 2 mRNA (mean ratio 0.86; p=0.006). Increased relative expression of 17HSD 2 mRNA was also significantly associated with both ER-negativity and progesterone receptor (PgR) negativity (mean ratios 0.27 and 0.22, respectively).
respectively; \( p<0.05 \). 17HSD 2 mRNA levels did not significantly correlate with tumor grade, tumor size, tumor stage, presence of HER-2 overexpression, S-phase fraction, MIB-1, and DNA ploidy (Table II and data not shown). We also evaluated the relationship between relative 17HSD 2 expression and disease recurrence and overall survival and found no significant correlations (data not shown).

**Association between 17HSD 2 and ER status in a large public dataset.** After searching the National Library of Medicine Gene Expression Omnibus Database for other datasets that included AA breast cancer cases run on the Affymetrix U133 platform, we identified the expO breast cancer database. This publicly available database contained information on 227 breast cancer specimens obtained from Cau and AA women (222 Cau, 15 AA). Information available for these patients included microarray signals and clinical information, such as age and ER status. We analyzed this dataset to search for associations between 17HSD 2 signal intensity and patient race, age, or ER status. We chose to evaluate age and ER status as these variables were found to significantly correlate with 17HSD 2 expression in our clinical series.

In this dataset, the average 17HSD 2 signal was 160 among the Cau patients and 170 among the AA patients (\( p=0.914 \) (Table III). There was a trend towards an association between younger age at diagnosis and increased 17HSD 2 signal (\( p=0.059 \)). There was a significant correlation between 17HSD 2 signal intensity and ER-negative tumors. The average signal intensity for 17HSD 2 was 70 in the ER-positive tumors and 338 in the ER-negative tumors (\( p<0.001 \)). There was no significant difference in 17HSD 1 signal intensities between AA and Cau patients (data not shown). The ratio of 17HSD 2/17HSD 1 signal was also significantly associated with ER status (\( p<0.001 \)) (data not shown).

**Discussion**

We began this study to identify genes that could potentially explain the aggressive clinical behavior of breast cancers that develop in AA women. In this study, we evaluated the expression of more than 22,000 genes in cell lines derived from AA and Cau patients. We performed a class comparison analysis to identify genes with significant differences in expression between these two groups and found that 17HSD 2 had significantly higher expression in the cell lines derived from AA patients. Real-time PCR analysis confirmed this difference in expression of 17HSD 2 among the cell lines. We next looked at a small clinical series of 16 breast cancer cases. While increased expression of 17HSD 2 among AA cases did not reach statistical significance in this series, 17HSD 2 expression was found to be significantly associated with younger patient age at diagnosis and with ER-negative and PgR-negative tumors. Finally, we looked at 17HSD 2 microarray signal intensities in a public clinical dataset. In this dataset, we could not confirm an association between 17HSD 2 and AA race; however, we found a trend towards an association between younger patient age and increased 17HSD 2 signal. 17HSD 2 signal was also found to be highly predictive of ER status in both AA and Cau patients.

The results of our cell line studies are in agreement with previous reports, although ours is the first study to investigate 17HSD 2 expression according to race. Liu et al. reported a low level of expression of 17HSD 2 in the Cau cell line MCF7 (24). Although several studies (25-27) have reported increased 17HSD 2 activity in the Cau cell line MDA-MB-231, our real-time PCR data indicate that of the Cau cell lines, MDA-MB-231 has the highest level of 17HSD 2 expression, although this...
level remains below that of the three AA cell lines. Of note, previous studies that evaluated 17HSD 2 expression in MDA-MB-231 did not compare expression in this cell line to that in cell lines derived from AAs.

We were unable to demonstrate the relative overexpression of 17HSD 2 among AA breast cancer cases in our clinical series or in the expO dataset. This is likely due to the very small sample sizes – there were only 11 AA cases in our series and only 15 AA cases in the expO dataset. Furthermore, there is currently much controversy regarding the ideal means for classifying and interpreting race in biological studies (28, 29).

In our dataset, race was determined by patient and/or physician report, a method that has been criticized as leading to considerable heterogeneity in the AA patient group. Such heterogeneity could contribute to the variability in 17HSD 2 expression among the AA cases.

Recent studies of 17HSD 1 and 17HSD 2 suggest that these enzymes play an important role in breast cancer development. 17HSD enzymes are expressed in epithelial tissues and catalyze the interconversion of estrogen between the inactive form estrone (E1) and the biologically potent form estradiol (E2). 17HSD 1 is responsible for the reduction of E1 to E2, and 17HSD 2 catalyzes the oxidation reaction of E2 to E1. 17HSD 2 appears to be the predominant form in plasma (30, 31), and may reduce the level of active estrogen in the circulation, thereby protecting tissues from the excessive influence of estrogen. In postmenopausal women, it has been demonstrated that in situ synthesis of E2 within the breast parenchyma predominates over uptake of E2 from plasma, providing for the maintenance of high levels of active E2 concentrations within breast tissue (32, 33). 17HSD 1 appears to be responsible for this production of active E2 within breast tumors in postmenopausal patients (30, 34).

In ER-positive breast cancer, E2 is a key growth and mitogenic factor, and studies of the 17HSD enzymes have led to the hypothesis that a predominance of 17HSD 2 may limit the availability of active estrogen in breast cells, leading to decreased estrogen-dependent proliferation of breast tissue (35). The oxidative pathway, catalyzed by 17HSD 2 in which E2 is converted to E1, has been found to be preferential in hormone-independent breast cancer cell lines, while in hormone-dependent cell lines the 17HSD activity is oriented in the reductive direction (25, 26, 35). However, the relationship between 17HSD 1 and 17HSD 2 enzyme expression and ER status in clinical breast cancer specimens is not so clear. Our examination of a series of breast cancer cases and of the expO database revealed the new finding of a significant association between 17HSD 2 relative expression level and ER-negativity, which is consistent with previous cell culture-based reports. Analysis of the expO dataset demonstrated that both high levels of 17HSD 2 and high ratios of 17HSD 2 to 17HSD 1 are highly predictive of ER status. Our findings are also consistent with a study by Gunnarsson et al., who demonstrated using real-time PCR that loss of 17HSD 2 mRNA was more frequent in ER-positive than ER-negative tumors (36). However, other reports have demonstrated positive, inverse, and no significant associations between 17HSD 1 expression and/or 17HSD 2 expression and ER status (37-39). These inconsistencies likely result from methodological differences in these studies – immunohistochemistry versus real-time PCR – and the influence of local, metabolic, and experimental conditions, including the concentration of cofactors (e.g., NADPH or NADP) and substrate and pH, on the orientation of 17HSD enzymatic activity (40).

In this study, we also demonstrated for the first time a strong inverse association between 17HSD 2 expression and age at diagnosis (mean ratio 0.86, p=0.006). The predominance of 17HSD 2 relative to 17HSD 1 has been postulated to play a role in the increased frequency of ER-negative tumors that develop in premenopausal patients. 17HSD 2 mRNA has been detected more commonly in premenopausal than postmenopausal cases of breast cancer (39). Conversely, Miyoshi et al. found that 17HSD 1 mRNA expression levels were significantly higher in postmenopausal than premenopausal breast cancers and that intratumoral E2/E1 ratios were significantly higher in postmenopausal breast cancers (34). Higher expression of 17HSD 2 among premenopausal patients may thus lead to the higher frequency of ER-negative tumors in this group. African-Americans, like premenopausal women, suffer from a higher frequency of ER-negative breast cancers. Thus, an overexpression of 17HSD 2 among AA women, as suggested by our cell line data, may provide a potential mechanism for this difference in prevalence.

Our study, in identifying a higher expression level of 17HSD 2 in AA breast cancer suggests a novel biological difference that may contribute to the more aggressive phenotype of AA breast cancer. It may also explain why AA women tend to develop more ER-negative breast cancers at a young age, compared to Cau women. Understanding the biological basis for differences in tumor biology between AA and Cau patients may have important implications for the treatment and prevention of breast cancer in both populations.

Acknowledgements

We thank Martha Neagu and Jennifer K. Drahos for assistance with the real-time PCR experiments, Richard Friedman for advice on gene expression analysis, and Anthony W. Ferrante and I. Bernard Weinstein for advice on study design. We are indebted to the Avon Foundation-funded Columbia University Macromolecule Breast Tissue Bank for the tissue specimens and to the International Genomics Consortium Expression Project for Oncology for access to their clinical datasets.
Grant support: Jean Sindab African-American Breast Cancer Project (A.K. Joe), Long Island University/Herbert Irving Comprehensive Cancer Center Minority Institution/ Cancer Center Partnership (CA91372, CA101388, CA101598) (A.K. Joe), and NIH grant T32CA09529 (R. Yaeger).

References


Received August 30, 2007
Accepted September 12, 2007