

Overall Study Design

The overall goal of the project is to investigate possible ethnic differences in gene expression in breast cancer when patient samples are matched for age, stage of disease and hormone receptor status. For each sample normal tissue from the same woman is used as a control to evaluate gene expression from the tumor tissue.

The final study will include 10 each African-American, Hispanic white and non-Hispanic white (Caucasian) women.

The study was originally designed to use fresh tissue samples but with the advent of the Breast Cancer DSA™ Research Tool has changed to using Formalin Fixed Paraffin Embedded (FFPE) samples.

Patient Study Criteria:

- Age 60 years or less
- No exposure to chemotherapy
- Triple Negative hormone receptor status

Almac Diagnostics Cancer DSA™ research tools

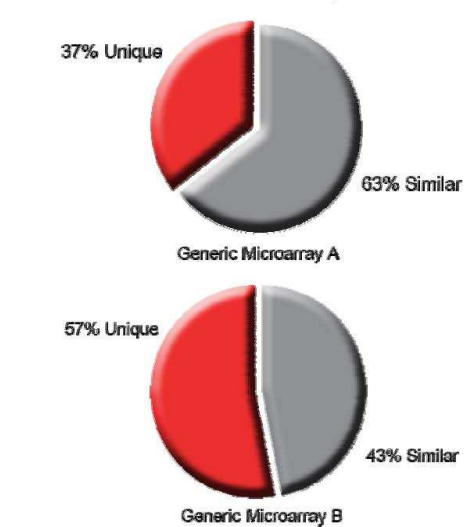
Standard microarrays provide large quantities of information, but are broadly representative of the human genome rather than a particular disease state.

The Breast Cancer DSA™ was developed by a process of high throughput sequencing, gene expression profiling and bioinformatics analysis, to fully characterize the transcriptome of disease and normal tissue. Therefore, it includes significant additional relevant data not available from other microarrays.

The Breast Cancer DSA™ research tool contains approximately 60,000 transcripts and is manufactured on Affymetrix GeneChip® technology.

Sequence analysis comparing the Breast Cancer DSA™ research tool with two leading commercially available generic microarrays clearly highlights the extent of its unique content as demonstrated in the figure below.

Almac Diagnostics Breast Cancer DSA™ Research Tool Compared with Two Commercially Available Generic Microarrays



Comparison of Breast Cancer DSA™ to the RefSeq database showed that 51% of the Breast Cancer DSA™ content is not present in the RefSeq database and 8% of the content represents antisense sequences to annotated transcripts.

Technical Specifications of the Breast Cancer DSA™ Research Tool	
Number of transcripts	~ 60,000
Feature Size	11 micron
Probe Length	25-mer
Probe Pairs/Probeset	11
Normalisation Ctrl Probesets	100
Hybridisation/Housekeeping Ctrl Probesets	62

Breast Cancer DSA™ and detection of differentially expressed transcripts

Technical assessment: Use with FFPE

The number of transcripts that were called present and were above the background in both RNAlater and FFPE samples was determined as shown in the table below.

	Transcripts detected in RNAlater	Transcripts detected in FFPE	Number of FFPE detected transcripts also detected in RNAlater
Breast Cancer DSA™ Research Tool	28,716	18,830	18,032
Affymetrix HG-U133 Plus 2	26,473	11,709	11,441

The high degree of data retention clearly demonstrates the power of the Breast Cancer DSA™ research tool when used in FFPE studies.

Use of the Breast Cancer DSA™ research tool in detection of differentially expressed transcripts. In most experimental studies, the end point of the analysis is the detection of differentially expressed transcripts. Analysis was carried out to determine the number of differentially expressed transcripts between normal and tumour tissue in our example study, defined as transcripts called present and above background with a fold change greater than 2 standard deviations of the mean with p-value <0.05. As can clearly be seen a large number of differentially expressed transcripts are detected in this experiment using both RNAlater and FFPE extracted samples. It is important to note that this experimental design is based on matched tumour and normal tissue from a single patient.

ACKNOWLEDGEMENTS: This project has been generously supported by a grant from the Susan G. Komen Breast Cancer Foundation, Grant No. POP0601150 and the Women's Cancer Association, University of Miami Miller School of Medicine. We also thank the clinicians who contributed to this project. We are continuing this project under a newly awarded DOD Synergy Grant.

Current ER-/PR-/Her2- Breast Cancer Patients— FFPE Samples

Ethnicity	Normal	Tumor
African-American women (AA)	10 samples	10 samples
Caucasian women (Cau)	8 samples	8 samples
Hispanic women (His)	10 samples	10 samples

Methods for RNA Extraction and Hybridization

Patient samples were obtained from the University of Miami Medical School Pathology Department, under IRB approval, as anonymous samples. All samples were from women less than 60 years of age and were known to be ER+/PR+/Her2-.

For each patient, pathologists cut new sections from paraffin-embedded sample blocks for normal tissue and for tumor tissue. Samples were sent to Almac Diagnostics for processing and hybridization to the Breast Cancer DSA™ Research Tool.

The following steps were performed by Almac Diagnostics:

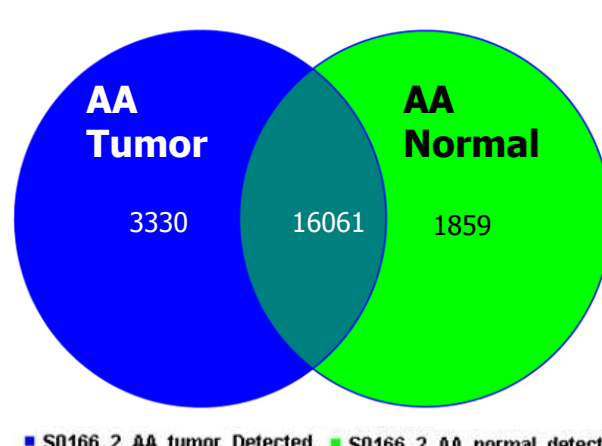
- Isolation of RNA from each sample
- RNA Amplification using the NuGEN FFPE System
- Generation of First Strand cDNA from 10-80 ng of total RNA
- Generation of a DNA/RNA Heteroduplex Double Strand cDNA and amplification
- cDNA Fragmentation and Labelling using a NuGEN System
- Affymetrix Hybridisation Washing, Staining and Scanning Protocol applied to the Breast Cancer DSA™ Research Tool.

Detection of Expressed Genes in Normal and Cancer FFPE Breast Samples by BC DSA

Number of Transcripts Detected by Ethnic Group

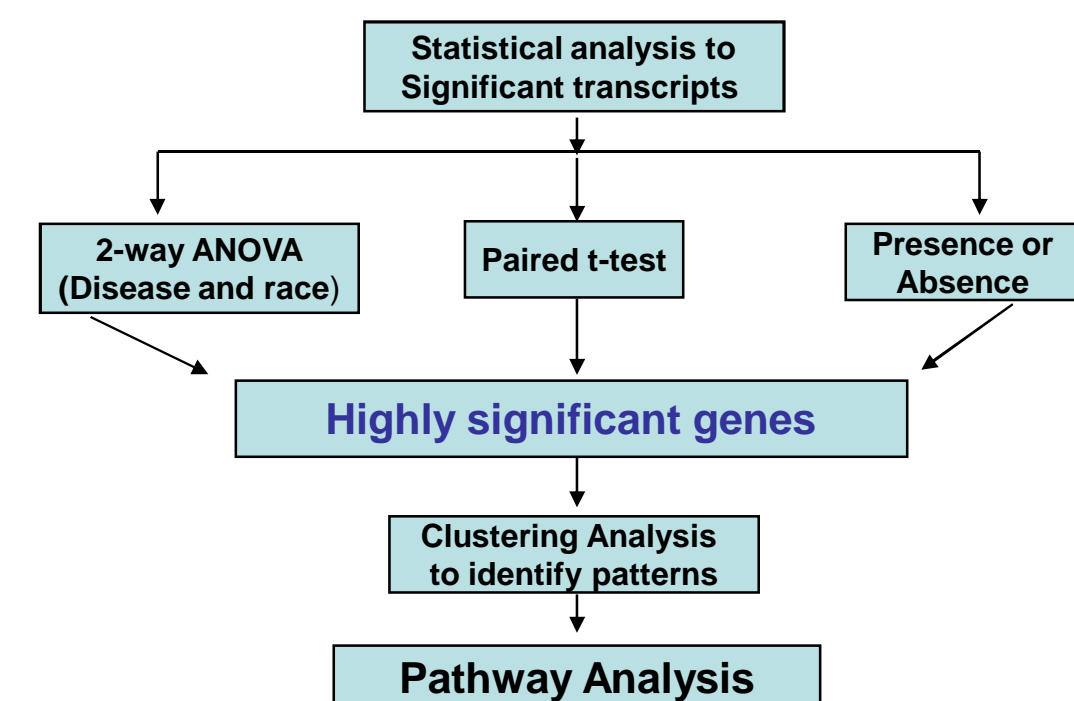
	AA	CAU	HIS
Detected in Tumor	3330	4164	4727
Detected in Normal	1859	1255	841
Detected in Both	16061	14504	10182

Numbers of Expressed Genes Detected in Breast Cancer Tumor FFPE Samples

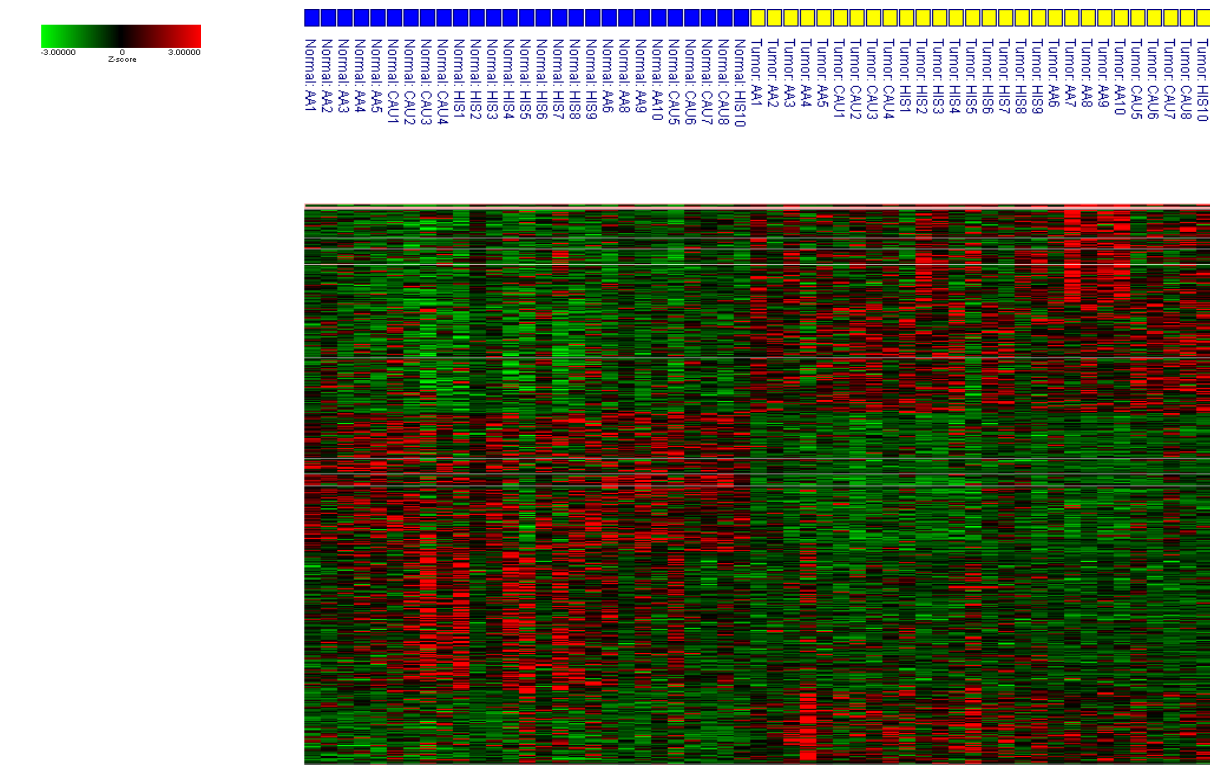


21250 transcripts are expressed on the Breast Cancer DSA with intensity significantly above background. Data from self-matched tumor and normal breast tissue was compared for the three ethnic groups. There are some genes (several hundreds to over a thousand) are detected in one or two ethnic groups.

Summary of Data Analysis Strategy



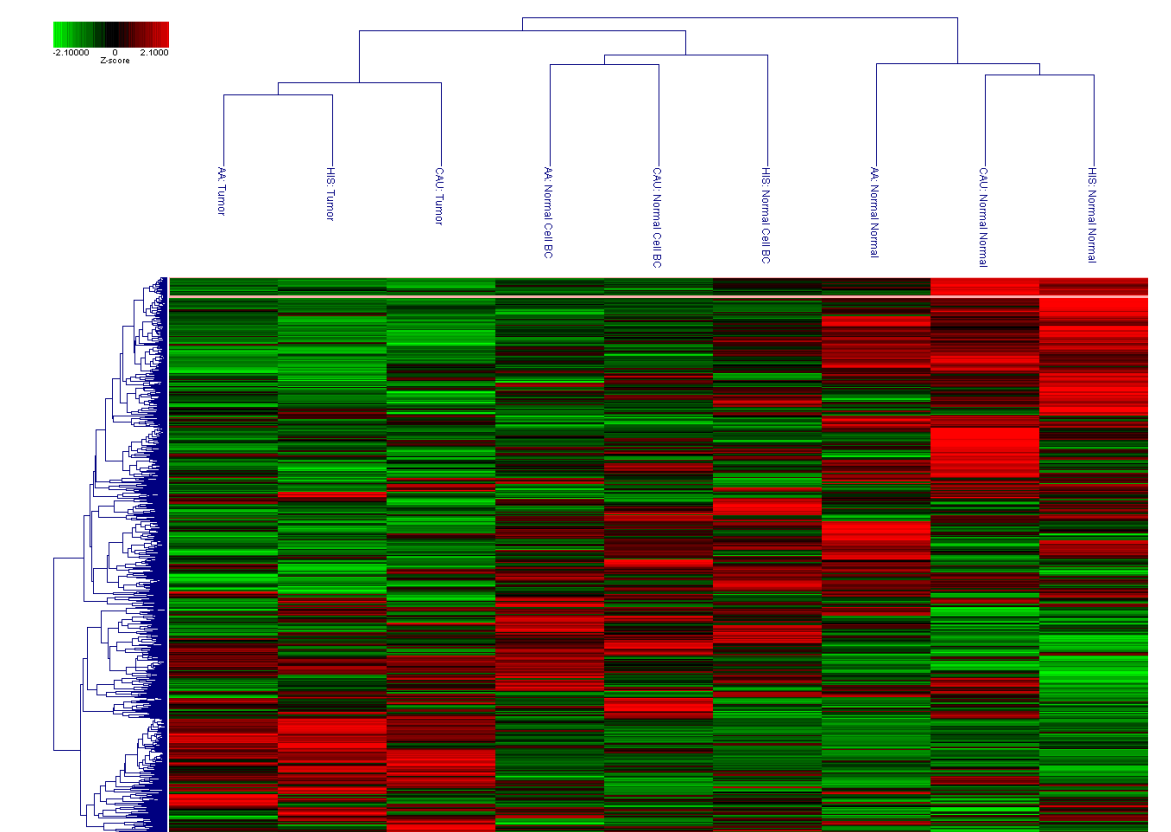
Cluster Analysis of Individual Tumor and Matched Normal Samples from Patients of Three Ethnicities



> 2-dimensional K-Mean Clustering of 28 patient samples, 10 African-American, 10 Hispanic and 8 Caucasian.

> Two main dendrogram groupings are seen: on the left (blue samples) self-matched normal samples, on the right (yellow samples) the tumor samples. Note the similarity of heatmaps of individual samples within a dendrogram group.

Cluster Analysis of Patient Samples from Three Ethnicities with Normal Breast Tissue Samples



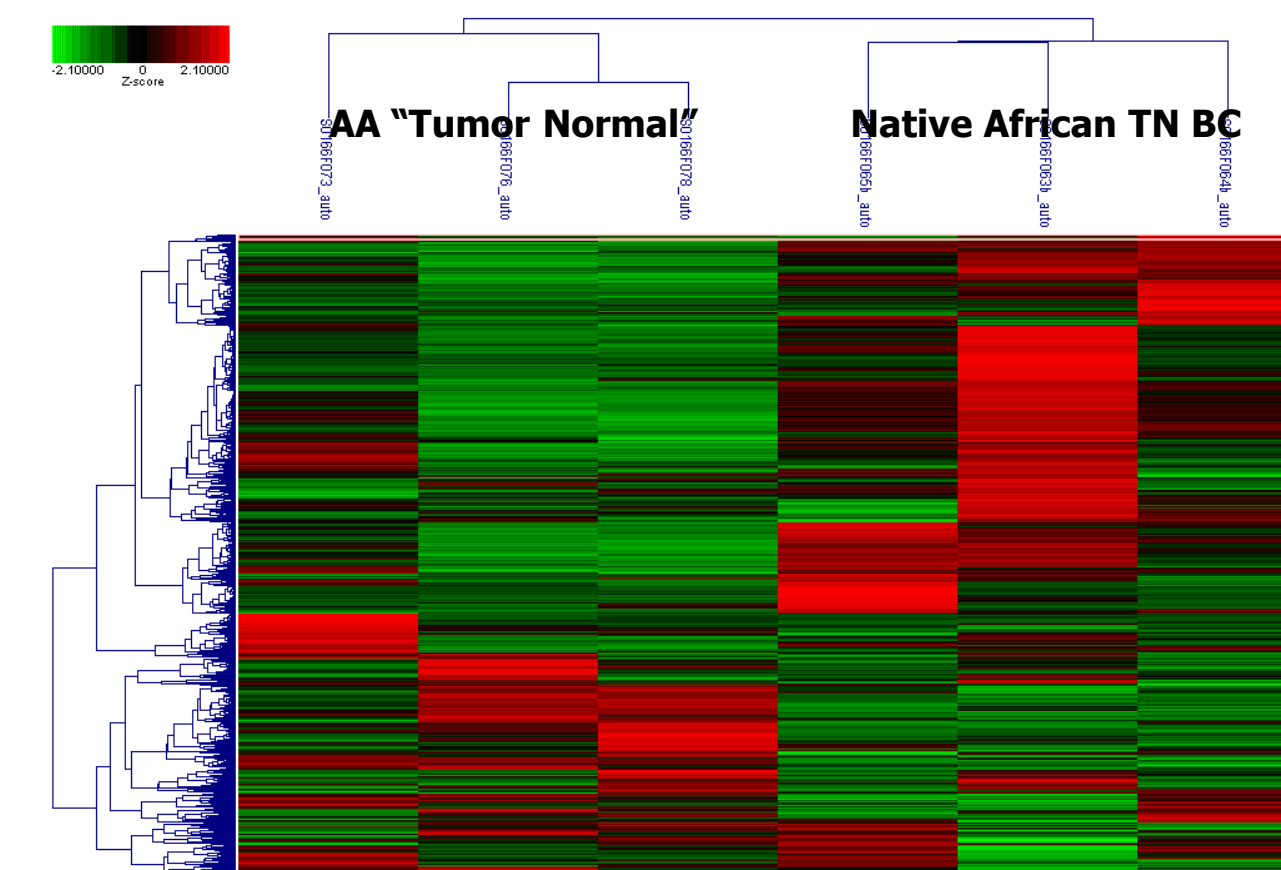
> Three main dendrogram groupings of transcripts are seen: on the left are the clustered tumor samples, second (the middle group) are the self matched normal cells adjacent to the patient tumor cells (termed "tumor normal"), lastly on the right is a cluster of "normal normal" samples obtained from non-cancer patients undergoing reduction mammoplasty. (up-regulation of genes red and down-regulation green).

> "Normal Normal" samples included 4 African-American, 3 Hispanic and 3 Caucasian samples.

> The 2-dimensional clustering of intensity data included 1442 probe sets selected based on their intensity (intensity > Background + 3stdev, p-value <= 0.01), 2-way ANOVA p* < 0.01 (With MTC by Benjamini-Hochberg FDR).

> Although "tumor normal" cells appear to be normal by pathology, it is apparent that gene expression alterations have occurred when compared to "normal normal" tissue from non-cancer patients.

Native African Triple Negative Breast Cancer Comparison with Normal Breast Tissue Samples



> A limited test set of triple negative breast cancer samples were obtained from collaborators at the Kijabe Kenya Hospital.

> Presented here are results from analysis of the first three native African samples. Initial comparison shows that high quality RNA can be extracted from the Kijabe samples.

> All samples were anonymous FFPE samples, hormone receptor status was verified by UM Pathology.

> An analysis of additional Kijabe TN BC samples with African American "tumor normal" and "normal normal" tissue samples is underway.

Summary and Conclusions

> Gene expression differences have been demonstrated to exist between BC "tumor normal" and "normal normal" tissue; suggesting that the tumor microenvironment has a strong influence upon surrounding "normal" cells.

Current Status of Project:

- We are selecting approximately 10 differentially expressed genes for validation by qRT-PCR, and we are extracting DNA from a subset of these specimens for hybridization to high-density SNP arrays, to assay possible DNA copy number variations (CNV's) and/or LOH in tumor samples.
- We are currently adding triple negative breast cancer samples from native Africans (Kijabe, Kenya) to the study. Initial studies show that high quality RNA can be obtained from the samples, and show gene expression difference from African-American samples.
 - The ability to obtain high quality RNA expression data from FFPE samples, illustrated here, offers new possibilities for genetic studies. Completion of this study should result in significant findings regarding genome-wide alterations associated with BC in several ethnic/racial groups, and increase understanding of the biological basis of ethnic-specific disparities in BC occurrence, mortality and therapeutic response.