Expression between African American and Caucasian Prostate Cancer Tissue Reveals that Stroma is the Site of Aggressive Changes

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Novelty and Impact: Novel to this study is the significant association of differential gene expression patterns with the tissue-type composition of prostate cancer. We are the first to associate gene expression to tumor *vs.* stroma tissue in prostate cancer of African American and Caucasian patients.

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Abstract

In prostate cancer, race/ethnicity is the highest risk factor after adjusting for age. African Americans have more aggressive tumors at every clinical stage of the disease, resulting in poorer prognosis and increased mortality. A major barrier to identifying crucial gene activity differences is heterogeneity, including tissue composition variation intrinsic to the histology of prostate cancer. We hypothesized differences in gene expression in specific tissue types would reveal mechanisms involved in the racial disparities of prostate cancer.

We examined seventeen pairs of arrays for African Americans and Caucasians that were formed by closely matching the samples based on the known tissue type composition of the tumors. Using pair wise T-test we found significantly altered gene expression between African Americans and Caucasians. Independently, we performed multiple linear regression analyses to associate gene expression with race considering variation in percent tumor and stroma tissue.

The majority of differentially expressed genes were associated with tumor-adjacent stroma rather than tumor tissue. Extracellular matrix, Integrin family and signaling mediators of the epithelial-to-mesenchymal transition pathways were all down regulated in stroma of African Americans. Using MetaCore (GeneGo Inc.) analysis, we observed that 35% of significant ($p < 10^{-3}$) pathways identified EMT and 25% identified immune response pathways especially for Interleukins -2, -4, -5, -6, -7, -10, -13, -15 and -22 as the major changes. Our studies reveal that

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altered immune and EMT processes in tumor-adjacent stroma may be responsible for the aggressive nature of prostate cancer in African Americans.

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Introduction

Prostate cancer is the most common cancer and the second leading cause of cancer deaths among men in all racial groups. Race/Ethnicity is the second highest risk factor for prostate cancer [1]. Incidence and mortality rates for African Americans (AA) are 1.5 and 2.3 times higher than for Caucasians (CA), respectively. AAs also have a higher incidence earlier in life and, upon diagnosis, present with a more aggressive disease [1]. Many explanations for health disparities have attributed them to modifiable factors such as low-socio-economic status and lack of access to health care; however, when such factors are controlled for, biological and/or genetic factors remain [1, 2].

AAs present with prostate cancer at a younger age, have a greater tumor volume for each clinical stage recorded, have greater PSA levels, and a more aggressive cancer for Gleason Score of 8 or greater, as compared to CAs [2, 3]. More recently, studies into biological mechanisms have uncovered genetic differences in p53 and BCL-2 between between AA and CA prostate cancer patients [4, 5]. Recent studies using microarray technology on samples from both African- and European-Americans revealed a significant number of biological processes that were differentially expressed in prostate tumors, such as immune response, apoptosis, focal adhesion and the Wnt signaling pathway [6, 7]. In addition, chromosome-level differences have been found in prostate tumors of AAs and CAs [8]. Using BAC- and oligo-based aCGH arrays, Rose and colleagues identified 27 chromosomal regions with significantly different copy number changes between AA and CA prostate tumors that associated with gene

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expression changes. Ancestral genotyping of AAs has revealed increased risk susceptibility associated with changes on chromosome 12 in prostate cancer [9].

Genetic instability and heterogeneity of a tumor are classic hallmarks of cancer, and prostate tumors are among the most heterogeneous [10]. Such heterogeneity creates challenges in identifying a standard of biological factors that are associated with patient outcome [11, 12]. Although much work has been performed to identify genetic differences within the prostate tumor, gene expression studies have been unable to distinguish tumor-specific gene expression from that of its microenvironment. Tumor associated stroma plays a critical role in tumor progression [13-16]; the biological differences in this tissue have the potential to contribute to the racial disparities of prostate cancer. Therefore, identifying genes that associate with stroma *versus* tumor tissue may help identify differences that play a role in racial disparities as compared to those that are a result of the genetic heterogeneity of prostate tumor tissue.

It was previously reported that changes in RNA expression via microarray are statistically reliable to distinguish normal stroma from tumor-adjacent stroma [16]. Through the use of linear regression modeling, RNA expression can be associated with cell-type specific tissue from array data of prostate tumor samples. This work has led to classifiers that are useful for the diagnosis of prostate cancer of stroma-rich biopsies, even in the case where the percent tumor tissue is extremely low in the sample. We have extended this modeling to understand the biological differences of prostate cancer by race using a subset of the U133A Affymetrix gene chip data (GSE08218) used in previous studies [16].

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For our analysis, we developed a combination of pair wise t-test between samples matched for tissue-type composition and multiple linear regression (MLR) models that identify differentially expressed genes between AA and CA patients and associate these expression changes with the percent tumor and stroma tissue found within the prostate cancer microarray samples. Our results show that specific processes related to the immune system and regulation of epithelial to mesenchymal transition (EMT) in the tumor-adjacent stroma are likely involved in the aggressive nature of prostate cancer in AA patients.

Materials and Methods

SPECS/EDRN Database

The NCI SPECS and EDRN consortium at UCI is an ongoing observational study that uses tissue and clinical data to generate gene signatures for the prognosis of prostate cancer [16-18]. As described previously, pathologic review was performed according to the TNM classification standard system [19]. Patient and clinical information was obtained through medical records and clinical pathologists verified the pathology of the tumor. Tumor volume was calculated using the volume of an ellipsoid (V=0.52 x length x width x height) [19]. Individual tumor histology sections were first evaluated by four pathologists for their percent of tumor, stroma, benign prostatic hyperplasia (BPH) and normal glands tissue before their use in the microarray [16, 18]. These percentages were averaged and used to associate gene expression with tissue type, as previously described [16]. In the current study, only percent tumor and stroma tissue were

considered, with regards to race of the patient, in the analyses to maximize the Goodness of fit in the MLR equations.

Data Normalization and Statistical Analysis

The SPECS U133A gene chip data was normalized with the plier algorithm using Expression Console software (Affymetrix, Santa Clara, CA). Normalized data were imported into R and SAS® software (v9.2, Copyright © 2002-2008, SAS Institute Inc., Cary, NC, USA) to perform the appropriate statistical analysis tests to identify significant gene expression differences by race [20]. The U133A Affymetrix gene chip for the SPECS study initially comprised of 148 arrays (GSE08218). The U133A chip data subset used in this study encompassed 82 arrays from 52 CAs and 17 arrays from 11 AAs patients (Table 1A). Clinical variables were tested for significant differences by race/ethnicity. Using the Wilcoxon-Mann Whitney test, there is no significant difference in the age of the patient at time of prostatectomy, pre-operative PSA and tumor volume between AAs and CAs (Table 1A). Gleason Score and TNM stage variables of AA and CA groups were compared by the Fisher exact test and revealed no significance between the two groups (Table 1A). Wilcoxon Mann Whitney test was also performed to determine statistical differences in the tissue types between AA and CA patients. There were no significant differences between the median values of percent tumor or stroma tissue in the AA and CA samples, although the variability in samples was quite high (Table 1B). Having patient samples that do not vary significantly in clinical variables allows for the study of differential gene expression by race without these factors confounding our results.

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To control for the variability in either percent tumor or stroma tissue in the samples, each AA patient array was matched with one CA patient array based on the percent tumor tissue recorded per chip. If no tumor tissue was identified then samples were matched by percent stroma tissue. These samples are identified either as “tumor-matched” or “stroma-matched” respectively. 16 tumor-matched (8 CA / 8 AA) and 18 stroma-matched (9 CA / 9 AA) array samples were analyzed separately using pair wise t-test. There were no statistical differences between clinical variables recorded in the matched samples. The similarity of these variables simplifies the formation of matched pairs based solely on tissue-type composition.

Pair wise t-testing was used to identify differentially expressed genes by race using the software BRB-Array Tools [21]. Genes were claimed as significant based on conventional criterions, i.e., the absolute $\log_2$ fold change (FC) was > 1.5 (equivalent to 2.8 FC in gene expression) and a $p < 0.05$. Similar criteria have also been used in “volcano plot” analyses [22] that aim to minimize false discovery. Genes with a $\log_2$ FC > 1.5 in CAs, as compared to AAs, are noted as being “upregulated” in CAs, where a $\log_2$ FC > 1.5 in AAs, as compared to CAs, are noted as being “upregulated” in AAs.

Independently, MLR analyses were performed using either the same samples used in matching (described above) or including the unmatched samples (all 99 arrays). The use of MLR models (Eqn. 1) allowed us to associate gene expression with tissue type [16-18]. The model assumes that total observed gene expression is a sum of the contribution from the different tissue types,

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$$Y_i = \beta_{i0} + \beta_{iT}X_T + \beta_{iS}X_S + \beta_{iR}R + \gamma_{iTR}X_TR + \gamma_{iSR}X_SR + \varepsilon_i \quad \text{(Eqn. 1)}$$

where Y_i is the $\log_2$ transformed observed probe set expression value for the ith probe; β_{i0} is the grand mean; Variables X_T, X_S and R represent tumor percent, stroma percent and race factor (0 for CA and 1 for AA) for a patient sample; β_{iT} and β_{iS} and β_{iR} are the expression coefficients associated with tumor percent, stroma percent and race factor, respectively, for probe i; γ_{iTR} and γ_{iSR} are the expression coefficients for interaction between tissue types and race factor for probe i: ε_i is normally distributed error. We are most interested in interaction terms which allows us to identify genes associated with tumor or stroma tissue that are also significantly different by race, i.e. significant γ_{iTR} and γ_{iSR}. Genes with significant γ values (by nominal criterion $p < 0.01$ to minimize false positives, type I error) were selected.

Significant genes found using pair wise t-testing of the matched samples were compared to those significant genes identified using MLR modeling of the *same* sample population. That is, the gene output using “tumor-matched” and “stroma-matched” samples in the pair wise t-test were compared to the gene output of those genes associated with the tumor*race interaction and stroma*race interaction variable in the MLR, respectively. To evaluate the significance of the overlap between the two tests, the observed overlap was compared to 10,000 random results generated by simulation in R [16]. T-testing ($p < 0.05$) allows us to identify a large set of genes that are differentially expressed by race by at least a $\log_2$ FC > 1.5, whereas the MLR modeling confirms this differential regulation by race with a more stringent criterion ($p < 0.01$) while also

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associating this differential gene expression to either percent tumor or percent stroma in the patient sample. The “tumor-matched” samples did not identify the same genes as the “stroma-matched” samples in the t-test. Similarly, genes associated with either percent tumor or percent stroma in the MLR did not overlap. Therefore, each differentially expressed gene, by race, associated with either tumor or stroma tissue, but not both.

Power Analysis

A power analysis was performed to determine if the matched samples were sufficient for finding differently expressed by race while minimizing type II errors. To determine the power of our paired t-test, we set a significance level for each probe at $\alpha = 0.05$, and an expression difference of $\log_2 FC = 1.5$ with the standard deviation of this difference being $\log_2 FC = 0.5$. Therefore, with 16 tumor-matched samples, we have a power of 99.9% to detect genes with at least $\log_2 FC = 1.5$. Likewise, with 18 stroma-matched samples, we have a power of 100% to detect genes with at least $\log_2 FC = 1.5$. In compensation for the false positives due to multiple comparisons, we applied the more stringent $\alpha = 0.0005$ in the power analysis, which leads to power of 91.4% and 97.8% for the tumor- and stroma-matched samples, respectively. Therefore, our paired t-test provides a power to reveal significant gene expression differences with at least $\log_2 FC = 1.5$, and we can conclude that our sample size is sufficient for this study. The power analysis was performed using the statistical software of R (http://www.r-project.org/).

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Gene Ontology Software

The Database for Annotation, Visualization and Integrated Discovery (DAVID) [23] and the Affymetrix online tool, NetAffx Analysis Center, were used to annotate the Affymetrix probes in our finalized gene list. Those probes that did not identify with genes were deleted from further study. Less than 2% of the probes could not be identified.

Using the Metacore software (GeneGo.Inc), an enrichment analysis was performed to identify significant biological pathways that existed in our gene list. These gene lists were categorized by race/ethnicity and tissue type to identify biological differences between AA and CA prostate cancers that associated with tumor or stroma tissue. To limit false discovery and increase biological significance, pathways of interest had to meet the following conventional criteria: FDR ≤ 5%, $p < 0.05$ and multiple genes (≥ 2) significantly associated with biological pathways in prostate tissue.

Results

African Americans and Caucasians exhibit significant differential gene expression.

For analysis of gene expression we had available 82 expression arrays of CAs but only 17 arrays of AAs. In order to enhance the sensitivity of a comparison and provide a validation criterion for the MLR results, seventeen pairs (n = 34) of AA and CA arrays were formed by carefully matching either

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percent tumor or percent stroma content for the arrays of each pair. As a measure of the efficacy of matching for a pair-wise t-test compared to a non pair-wise t-test of the *same* samples, we compared the standard deviations for the mean difference of percent tumor and stroma of the matched samples to the standard deviations of the difference of means of tumor and stroma percentages for the same, unmatched samples. Matching resulted in a dramatic decrease in the standard deviation of the mean difference for tumor-matched samples (σ matched = 0.6477985 *vs.* σ unmatched = 10.66495). Likewise, matching on percent stroma content greatly reduced the standard deviation of the mean difference (σ matched = 0.2939724 *vs.* σ unmatched = 7.269716). By matching samples based on percent tumor and stroma content we decreased the standard deviations by 93.9% and 96% respectively (Table 2). Pair wise t-testing identified 398 and 1016 probes for tumor-matched and stroma-matched samples respectively (Table 3).

In order to extend the number of cases analyzed we utilized a MLR analysis on the basis of Eqn 1 (Materials and Methods). First we sought to validate the MLR approach. For this step we used the 34 cases for which the pair wise t-test results were known and compared the MLR results for these cases to the pair wise t-test results. We used a reduced MLR model compared to eqn. 1 by dropping the race variable (β_R) and only considering the interaction between 'race' and tissue types – the principle goal. Using this reduced model and only the 34 matched-arrays, 127 probe sets were significantly associated with tumor tissue and differentially expressed by race ($p < 0.01$) (Table 3). Similar to the

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matched t-test results, 1264 probe sets were significantly associated with stroma tissue and differentially expressed by race ($p < 0.01$).

To evaluate this result we compared the overlap among the significantly, differentially expressed genes for the t-test and for the MLR model. 39 probe sets were identical between the tumor-matched arrays and 674 probe sets were identical between the stroma-matched arrays. These intersect numbers were compared to that expected by chance based on simulation studies. The percent overlap for both tumor and stroma cells were found to be significant ($p < 0.001$) (Table 3). Thus, the MLR method, applied to the limited sample of cases, significantly replicated the results for the matched t-test.

Since the percent overlap between genes discovered by the pair wise t-test and by the MLR procedure appeared valid, we adopted a “boot-strap” approach wherein the MLR method was used to introduce all the remaining CA samples each with known tissue-type composition. The MLR model was then extended to include all 99-sample arrays thereby providing an increase in the number of potential genes that are significantly differentially expressed. Indeed, using all 99 arrays, 542 probe sets were significantly associated with tumor tissue, and 2323 probe sets were significantly associated with stroma tissue, all differentially expressed by race (Table 3). Probes appearing in both the pair wise t-test and the MLR analysis had a significant overlap ($p < 0.001$). Probes lists from MLR models, matched (n=34) and unmatched samples (n=99) also significantly overlapped with each other (Table 4). Therefore, we extended our final gene list by including significant probes that appeared in both the pair wise t-

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test and either of the two MLR tests. This resulted in 63 probe sets from the tumor-associated samples representing 56 unique genes, and 818 probes from the stroma-associated samples that represented 677 unique genes (Table 4).

Tumor- and stroma-associated genes were separated by their $\log_2$ FC to identify the number of genes upregulated in CAs as compared to AAs. Interestingly, upregulated tumor-associated genes were evenly distributed between each group while most stroma-associated genes were upregulated in CAs. Further, there does not appear to be a significant difference in the number of genes identified using the matched *vs.* the unmatched samples (Table 4). Therefore, this discrepancy cannot be due to differences in the sample size of each test (n=34 *vs.* n=99), but rather the differences in the biological nature of stroma tissue of CAs *vs.* AAs.

Two genes in our list, phosphoserine phosphatase (PSPH) and crystalline beta 2 (CRYBB2), were previously shown to serve as strong biomarkers for identifying prostate cancer in AAs [6]. PSPH (probe 205048_s_at) has a $\log_2$ FC = 3.69 in AAs as compared to CAs, where CRYBB2 (probe 206777_s_at) had log2 FC = 2.54 in AAs as compared to CAs. Interestingly, we found their *differential* expression is significantly associated with stroma, not tumor, tissue, although this significance is not understood.

Functional relationships reveal differences in immune processes and regulation of EMT.

Functional relationships of the genes were assessed by computer-assisted searches using MetaCore software. Genes associated with tumor tissue

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were analyzed separately from those associated with stroma tissue. An advantage of MetaCore is that the underlying literature can be filtered by topic. The enrichment analysis was filtered for prostate tissue specific genes and only pathways with a significance level of $p < 0.05$, FDR ≤ 5% and with ≥ 2 genes identified have been listed (Table 5).

Tumor-associated pathways: 56 genes were found to be both differentially expressed between AA and CA men and associated with prostate tumor tissue. Four pathways were significantly associated with this gene list. Interestingly, 3 out of the 4 pathways are related to immune responses and one pathway was associated with cell adhesion (Table 5).

Stroma-associated pathways: 677 genes were found to be both differentially expressed between AA and CA men and associated with prostate cancer stroma tissue. 103 pathways were identified to be significantly associated with this gene list (Table 5). 19 of the 103 pathways were related to cytoskeletal remodeling, cell adhesion and regulation of EMT. Interestingly, of the top 20 pathways ($p < 10^{-4}$), 50% are associated with these three processes. Pathways involved with immune responses accounted for 20 out of the 103 pathways recorded, roughly 20%.

Differences in gene expression imply that immune response in both tumor and stroma may differ by race.

Three out of the four pathways identified by the MetaCore analyses in tumor tissue, and 20% of the pathways identified in stroma tissue are related to immune processes. Genes associated with these pathways are shown in Table 6.

Genes linked to antigen presentation (eg. HLA-DPA1 and HLA-DMB) are some of the most differentially expressed genes identified by our analyses ($\log_2$ FC of 7.14 and 3.23 respectively). In stroma tissue, the immune response pathways appear to be linked particularly to cytokine signaling, including pathways for interleukins -2, -4, -5, -6, -7, -10, -13, -15 and -22, as well as TGF-β, MIF and oncostatin M. These data strongly suggest that immune processes in both tumor and stroma tissue may be linked to racial disparities in prostate cancer.

Upregulation of Integrin signaling in Caucasians suggests stabilization of cell adhesion and stress fiber formation.

We found several pathways involved in cytoskeleton remodeling, cell adhesion and regulation of EMT that were differentially expressed in the stroma of AA and CA prostate cancers (Table 5). To increase the likelihood of biological significance, we chose to look more closely at only the Metacore pathways that included multiple (>10) genes from our list. The genes associated with these pathways are described in Table 6. Most of these were upregulated in CAs, many of which associated with Integrin mediated signaling[14, 24-27]. These factors include several integrins and their downstream targets, NCK2 (Grb2), ROCK2, Vinculin, α-Parvin and α-Actinin along with Arp2/3 in CA patients. Likewise, many ECM proteins that interact with the integrin receptors were identified, including Laminin-1, Collagen-I and IV, Fibronectin and Nidogen. In all, we found 15 probes corresponding to nine collagens, four probes for fibronectin, four probes corresponding to three laminins, and six probes corresponding to six integrins. All of these products suggest the stabilization of stress fiber formation and cell

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adhesion sites in CAs [24, 28, 29]. We also found VE-cadherin upregulated in CAs, suggesting possible cadherin-mediated cell adhesion as well [26]. In addition, the up-regulation of Elastin, BMP-4 and the Fibulins-2 and -5 in AAs support that differences in expression patterns of ECM proteins and their downstream signaling effectors may play a role in racial disparities of tissue remodeling and EMT in prostate cancer. It is important to note that of the 86 probes identified from these pathways, 82 (95%) of them were found using the matched samples (n=32) and 92% of them were found using the unmatched samples (n=99). Therefore, our results are consistent regardless of how we choose to analyze the patient population.

Discussion

There is a well-documented racial disparity in prostate cancer, with AA men having significantly higher incidence and mortality rates as compared to CAs [1]. In this study, microarray data previously obtained in the SPECS/EDRN study have been analyzed using new methods to reveal gene expression differences by tissue type (tumor *vs.* stroma) in order to elucidate biological mechanisms of racial disparities. In all, 733 genes have been found to have differential expression in our samples, 56 associated with tumor tissue and 677 in stroma tissue. In fact, two biomarkers previously described for identifying prostate cancer in AAs [6], PSPH and CRYBB2, were found to be associated with stroma tissue, a fact not revealed by earlier studies. It is interesting, although not

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surprising, that the overwhelming majority of these genes are differentially expressed in stroma tissue, as stroma tissue is a major player in tumor biology.

Surrounding stroma tissue heavily influences tumorigenesis. In turn, as tumors become more aggressive they have a stronger impact on changes within the surrounding stroma tissue [13]. We have found many biological processes associated with stroma tissue to be differentially expressed between AAs and CAs. Our findings suggest that tumor-associated stroma may help contribute to the racial disparities observed in prostate cancer.

Genes involved in stabilization of stress fibers and cell adhesion sites are upregulated in Caucasians

Tumor-adjacent stroma tissue has very different properties from normal epithelium. Differences in gene expression patterns in tumor-adjacent stroma directly influence the growth and progression of the tumor [25]. These tissues work together *via* physical interactions in the extracellular matrix (ECM). Cytoskeletal remodeling, cell adhesion and EMT pathways play a critical role in cell migration and metastases; 90% of cancer mortality cases are a direct result from metastasis [25, 30, 31 13].

Cell adhesion is important not only for the maintenance of epithelial tissue but it can also serve as a scaffold for cell migration, a mechanism essential during metastasis. In normal epithelial tissues, cell migration is limited and any migrating epithelial cells would be quickly eliminated via the body's natural defense mechanisms [32]. This environment is much different in cancer. An

inflammatory, stromal microenvironment surrounds the tumor and allows cancerous cells to escape these regulatory controls [13, 32, 33].

We have found many genes associated with the stability of cell adhesion sites and stress fiber formation via Integrin signaling differentially expressed in CA prostate samples as compared with our matched AA samples. These include NCK2, ROCK2, Vinculin, PARVA, ACTN, ARP2/3 and NID1, PPARD and TCF4 as well as multiple laminin, fibronectin, collagen and integrin genes. Decreased cell adhesion and disrupted extracellular matrix is a hallmark of EMT [10]. The concordant decrease in expression of all of these genes in the stroma of AA suggests an increased extent of EMT. Indeed over a third of the significant pathways identified here (Table 5), including the five *most* significant, are for EMT mechanisms. These observations are consistent with the impact stroma tissue has on the aggressive nature of adjacent tumor as well as the clinical evidence that AAs present with a more aggressive cancer upon diagnosis [2, 13]. In particular, we suggest that a more aggressive form of stroma-associated EMT compared to CAs is one basis for the racial disparities of prostate cancer.

Differences in immune response may play a role in racial disparities

We have found many specific immune response pathways associated with tumor as well as stroma tissue of the prostate. For example, HLA-DMB is upregulated in tumor tissue of CA patients in our sample (Table 6). This expression has been previously shown to positively correlate with increased CTL infiltration and improved prognosis in ovarian cancer [34]. We also found upregulation of another MHC class II molecule in CAs, HLA-DPA1. Its role in

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tumor-immunology is undefined but it would be interesting if its expression also correlated with improved prognosis in cancer. In addition, 20% of all significant pathways from the Metacore analysis (Table 5: Stroma-associated) implicate immune response differences in stroma tissue. In particular, cytokine signaling including the interleukin-mediated pathways of IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-13, IL-15 and IL-22, as well as TGF-β, MIF and oncostatin M, appear to be altered by race in these prostate cancer samples. These data are consistent with previous observations by Wallace et al [6] but their studies did not differentiate immune gene expression by tissue type.

Interestingly, when we analyze the genes that Wallace and colleagues reported with the Metacore software, and compare these pathways with the pathways from our studies, we find a 31% overlap between the two groups. Of the 23 total immune response pathways we found, 13 of these, roughly 56%, are also found using the Wallace data. Taken together, our data suggest that immune processes may significantly differ in both tumor and stroma tissue between AA and CA and their role in racial disparities is worth further evaluation. Both tumor and stroma associated immune response and inflammation responses plays an important role in tumorigenesis [2, 13, 25].

Limitation in the study

The study was limited by the small number of AA patients. In order to power up the statistical analysis we used a pair wise t-test to even out potential confounding factors between AA and CA patients, which helped us identify gene expression changes only due to race. The same matched data were also

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analyzed using an independent MLR analysis, yielding significantly similar results. The small-scale MLR analysis using matched samples justified the MLR analysis using entire patient samples by which we identified interesting gene changes between AA patients and CA patients.

In spite of the small sample size, our results support previously published studies as well as the hypothesis that pathways associated with immune/inflammatory processes as well as those regulating EMT may vary by race and emphasize that these changes arise in tumor-adjacent stroma. These differences in stroma tissue could influence the frequency of development and increased aggressiveness of prostate cancer in AAs. Further investigation of the tumor microenvironment of prostate cancer of AAs is warranted as an understudied source of new information about the biology of prostate cancer in AAs.

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Table 1A. Descriptive Statistics of U133A Patient Cohort

Characteristic	N	Caucasian	African American	p-value
#patients	63	52	11	-
Median age (yrs)	63	63.0	60.0	0.0582
Gleason Score	63			0.3065[a]
less than 7		24	3	
equal to 7		18	7	
greater than 7		10	1	
Stage:	61			0.7264[a]
≤pT2 (n)		35	7	
≥pT3 (n)		15	4	
Surgical Margins	63			0.4799[a]
negative		35	9	
positive		17	2	
PSA	62	9.3	8.0	0.9120
Median tumor volume (cm^3)	54	41.2	33.8	0.6640

Table 1B. Descriptive Statistics of the Tissue Percentages in U133A Array

Descriptive Stastistics of Total U133A Array

Characteristic	N	Caucasian	African American	p-value
#Array files	99	82	17	
#Tumor Arrays	58	50	8	
Median tumor %		43.5	41.5	0.4913
Median stroma %		45.5	47.0	0.7351
#Stroma Arrays	41	32	9	
Median stroma %		77.0	70.0	0.5284

Descriptive Stastistics of Matched U133A Array

Characteristic	N	Caucasian	African American	p-value
#Array files	34	17	17	
#Tumor-matched Arrays	16	8	8	
tumor%: mean (st.dev) median		36 (21.4) 41.5	35.75 (21.3) 41.5	1.0000
stroma%: mean (st.dev) median		48.25 (18.4) 42.5	48.88 (17.8) 47.0	0.9162
#Stroma-matched Arrays	18	9	9	
tumor%: mean (st.dev) median		0 (0) 0	0 (0) 0	n/a
stroma%: mean (st.dev) median		70.8 (15.2) 70.0	71.2 (15.7) 70.0	0.8246

(1A) Descriptive statistics of the clinical variables in the Affymetrix U133A gene chip data using Wilcoxon Mann Whitney and [a]Fisher exact test were used to report significant differences between the two groups. (1B) Descriptive statistics of the cell-type composition in the Affymetrix U133A gene chip data using Wilcoxon Mann Whitney were used to report significant differences between the two groups. There were no significant differences between all arrays or the matched subset.

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Table 2. Comparison of Standard Deviations of the Differences of the means between matched vs unmatched samples.

Characteristic	matched	unmatched	% Reduction due to matching
"Tumor" Arrays (σ)	0.648	10.665	93.9%
"Stroma" Arrays (σ)	0.294	7.267	96.0%

High variabilty in percent tumor and percent stroma in the samples was controlled for by matching patient arrays based on percent tissue. Matching samples drastically reduced the standard deviations of the differences of the means among the samples.

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Table 3. Probes that differentially expressed between race and tissue type by two independent tests (matched arrays vs unmatched arrays).

	Pairwise t-test ($p < 0.05$, $\log_2 FC \geq 1.5$)	MLR (matched samples) ($p < 0.01$)	# Overlapping Probes	p-value
# Arrays tested	34	34		
Tumor associated probes	**398**	127	39	<0.001
Stroma associated probes	**1016**	1264	674	<0.001
	Pairwise t-test ($p < 0.05$, $\log_2 FC \geq 1.5$)	**MLR (total samples) ($p < 0.01$)**	**# Overlapping Probes**	**p-value**
# Arrays tested	34	99		
Tumor associated probes	**398**	542	31	<0.001
Stroma associated probes	**1016**	2324	720	<0.001

Tumor-matched probe output from the pairwise t-test was compared to probes significantly associated with tumor*race interaction term in the MLR. Likewise, Stroma matched probe output from the pairwise t-test was compared to probes significantly assoicated with stroma*race interaction term in the MLR. The MLR was run twice, once with the samples used for matching (top table) and a second time with all the arrays (bottom table). Genes were tested for significance of overlap between the two tests and only those genes that appeared in the t-test and either of the MLR tests were further evaluated.

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Table 4. Final List of Tumor and Stroma Associated Genes from Two Independent Tests.

	Overlapping probes (matched)	Overlapping probes (unmatched)	# Overlap between groups	Total Probes	Total Genes
Tumor associated probes	39	31	7	63	**56**
Stroma associated probes	674	720	576	818	**677**

Overlapping probes of both MLR analysis with the pairwise t-test contribute significantly to the total gene list. MLR using only the matching arrays (n=34) contribute 39 of the 63 total tumor associated probes probes, 7 of which overlap with the unmatched array analysis (n=99). Likewise, the matching arrays also contribute significantly to the stroma associated probes, 576 of which overlap with the unmatched array analysis. 63 probes associate with percent tumor tissue, where 818 probes associate with percent stroma tissue. Given there are multiple probes per gene in the U133A chip, this equates to 56 tumor associated genes and 677 stroma associated genes.

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Table 5. Significant Cytoskeletal remoding, EMT, Cell adhesion and Immune Response pathways from Metacore Analysis that are differentially regulated by race.

Significant Pathways of Tumor-associated genes with a $\log_2$ FC > 1.5 difference by race

Pathway	p-value	# Genes
Immune response_MIF - the neuroendocrine-macrophage connector	6.878E-05	3
Immune response_Antigen presentation by MHC class II	1.983E-04	3
Cell adhesion_Cell-matrix glycoconjugates	5.594E-04	2
Immune response_NF-AT signaling and leukocyte interactions	3.002E-03	3

Significant Pathways of Stromal-associated genes with $\log_2$ FC > 1.5 difference by race

Pathway	p-value	# Genes
Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodeling	3.874E-11	19
Cytoskeleton remodeling_Cytoskeleton remodeling	5.402E-09	16
Cytoskeleton remodeling_Integrin outside-in signaling	3.021E-08	11
Development_Regulation of epithelial-to-mesenchymal transition (EMT)	6.033E-08	12
Cell adhesion_Chemokines and adhesion	2.192E-07	14
Cell adhesion_ECM remodeling	5.719E-06	9
Development_WNT signaling pathway. Part 2	6.738E-06	9
Cytoskeleton remodeling_Fibronectin-binding integrins in cell motility	1.029E-05	7
Development_TGF-beta-dependent induction of EMT via MAPK	2.179E-05	8
Development_TGF-beta-dependent induction of EMT via SMADs	2.411E-05	7
Immune response_IL-13 signaling via PI3K-ERK	3.482E-05	8
Immune response_IL-7 signaling in T lymphocytes	4.238E-05	7
Immune response_Oncostatin M signaling via JAK-Stat in mouse cells	6.899E-05	5
Immune response_IL-7 signaling in B lymphocytes	9.705E-05	7
Immune response_Oncostatin M signaling via JAK-Stat in human cells	1.197E-04	5
Cell adhesion_Integrin-mediated cell adhesion and migration	1.989E-04	7
Immune response_IL-15 signaling via JAK-STAT cascade	2.440E-04	5
Some pathways of EMT in cancer cells	2.931E-04	7
Immune response_IL-10 signaling pathway	4.481E-04	5
Immune response_IL-6 signaling pathway	7.598E-04	5
Immune response_IL-13 signaling via JAK-STAT	8.284E-04	6
Immune response_IL-4 signaling pathway	8.284E-04	6
Immune response_IL-4 - antiapoptotic action	8.930E-04	5
Development_TGF-beta-dependent induction of EMT via RhoA, PI3K and ILK.	1.053E-03	6
Development_NOTCH-induced EMT	1.203E-03	4
Immune response_IL-22 signaling pathway	1.397E-03	5
Immune response_Gastrin in inflammatory response	1.851E-03	7
Immune response_MIF-JAB1 signaling	2.983E-03	4
Cell adhesion_PLAU signaling	2.993E-03	5
Cytoskeleton remodeling_FAK signaling	3.232E-03	6
Cytoskeleton remodeling_Role of PKA in cytoskeleton reorganisation	3.351E-03	5
Cytoskeleton remodeling_Neurofilaments	3.481E-03	4
Immune response_HTR2A-induced activation of cPLA2	4.607E-03	5
Immune response_IL-5 signalling	5.091E-03	5
Cell adhesion_Histamine H1 receptor signaling in the interruption of cell barrier integrity	5.611E-03	5
Immune response_IL-15 signaling	5.759E-03	6
NGF activation of NF-kB	6.034E-03	4
Immune response_Inhibitory action of Lipoxins on pro-inflammatory TNF-alpha signaling	6.760E-03	5
Immune response_ETV3 affect on CSF1-promoted macrophage differentiation	7.682E-03	4
Immune response_IL-2 activation and signaling pathway	8.064E-03	5

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Table 6. A Subset of genes associated with Metacore Pathways of Interest.

Genes Associated with Metacore Immune Response Pathways

Gene symbol	Probe ID	Gene name	Log_2 FC (CA/AA)
AKT3	212609_s_at	v-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)	1.82
BCL2	203685_at	B-cell CLL/lymphoma 2	1.85
CACNA1C	211592_s_at	hypothetical protein LOC100131098; calcium channel, voltage-dependent, L type, alpha 1C sut	6.25
CACNA1D	210108_at	calcium channel, voltage-dependent, L type, alpha 1D subunit	3.03
CCND2	200953_s_at	cyclin D2	2.44
ELAVL1	201726_at	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R)	1.89
FOXO1	202723_s_at	forkhead box O1	1.75
HLA-DMB	203932_at	major histocompatibility complex, class II, DM beta	3.23
HLA-DPA1	211990_at	major histocompatibility complex, class II, DP alpha 1	7.14
HLA-DQB1	211656_x_at	major histocompatibility complex, class II, DQ beta 1; similar to major histocompatibility comple	1.49
IL13RA1	201888_s_at	interleukin 13 receptor, alpha 1	1.64
IL4R	203233_at	interleukin 4 receptor	1.56
IRS1	204686_at	insulin receptor substrate 1	2.08
JAK1	201648_at	Janus kinase 1	2.13
KCNMA1	221583_s_at	potassium large conductance calcium-activated channel, subfamily M, alpha member 1	1.56
MCL1	200797_s_at	myeloid cell leukemia sequence 1 (BCL2-related)	1.54
OSMR	205729_at	oncostatin M receptor	1.85
PLA2G4C	209785_s_at	phospholipase A2, group IVC (cytosolic, calcium-independent)	1.64
REL	206036_s_at	v-rel reticuloendotheliosis viral oncogene homolog (avian)	1.82
SMAD3	218284_at	SMAD family member 3	1.89
SOCS5	209647_s_at	suppressor of cytokine signaling 5	1.54
SOS1	212780_at	son of sevenless homolog 1 (Drosophila)	1.92
STAT3	208991_at	signal transducer and activator of transcription 3 (acute-phase response factor)	1.72
TNFRSF1B	203508_at	tumor necrosis factor receptor superfamily, member 1B	1.92

Genes Associated with Metacore Cytoskeletal Remodeling, EMT and Cell Adhesion Pathway

Gene symbol	Probe ID	Gene name	Log_2 FC (CA/AA)
ACTN1	211160_x_at	actinin, alpha 1	2.50
ACTR2	200727_s_at	ARP2 actin-related protein 2 homolog (yeast)	1.67
BCL2	203685_at	B-cell CLL/lymphoma 2	2.27
BMP4	211518_s_at	bone morphogenetic protein 4	0.21
CALD1	201616_s_at	caldesmon 1	2.27
CCND1	208712_at	cyclin D1	1.85
CDH5	204677_at	cadherin 5, type 2 (vascular endothelium)	1.85
COL1A2	202404_s_at	collagen, type I, alpha 2	2.44
COL3A1	211161_s_at	collagen, type III, alpha 1	2.08
COL4A1	211981_at	collagen, type IV, alpha 1	2.78
COL4A2	211964_at	collagen, type IV, alpha 2	2.78
EDNRA	204463_s_at	endothelin receptor type A	3.33
EFNB2	202668_at	ephrin-B2	1.85
ELN	212670_at	elastin	0.62
EPHA2	203499_at	EPH receptor A2	2.13
FBLN2	203886_s_at	fibulin 2	0.60
FN1	211719_x_at	fibronectin 1	1.75
GRLF1	202046_s_at	glucocorticoid receptor DNA binding factor 1	1.69
ITGA2	205032_at	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	1.54
ITGA3	201474_s_at	integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	1.52
ITGA5	201389_at	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	1.64
ITGA7	216331_at	integrin, alpha 7	2.04
ITGA8	214265_at	integrin, alpha 8	2.38
ITGB3	204627_s_at	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	1.52
JAG1	209098_s_at	jagged 1 (Alagille syndrome)	2.08
LAMA4	202202_s_at	laminin, alpha 4	1.82
LAMA5	210150_s_at	laminin, alpha 5	2.08
LAMC1	200771_at	laminin, gamma 1 (formerly LAMB2)	2.70
MET	203510_at	met proto-oncogene (hepatocyte growth factor receptor)	2.22
MMP14	202828_s_at	matrix metallopeptidase 14 (membrane-inserted)	1.69
MSN	200600_at	moesin	1.85
MYH9	211926_s_at	myosin, heavy chain 9, non-muscle	2.13
NCK2	203315_at	NCK adaptor protein 2	1.75
NID1	202007_at	nidogen 1	2.70
PARVA	217890_s_at	parvin, alpha	1.85
PPARD	37152_at	peroxisome proliferator-activated receptor delta	2.50
RBP4	219140_s_at	retinol binding protein 4, plasma	0.14
REL	206036_s_at	v-rel reticuloendotheliosis viral oncogene homolog (avian)	1.82
ROCK2	202762_at	Rho-associated, coiled-coil containing protein kinase 2	2.63
SERPINE1	202627_s_at	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	3.57
SMAD3	218284_at	SMAD family member 3	1.89
SOS1	212780_at	son of sevenless homolog 1 (Drosophila)	1.92
SPTBN1	212071_s_at	spectrin, beta, non-erythrocytic 1	1.79
SRF	202401_s_at	serum response factor (c-fos serum response element-binding transcription factor)	2.08
STXBP1	202260_s_at	syntaxin binding protein 1	1.75
TGFB2	209909_s_at	transforming growth factor, beta 2	1.96
TGFBR3	204731_at	transforming growth factor, beta receptor III	1.79
THBS1	201109_s_at	thrombospondin 1	2.94
TRAF3	221571_at	TNF receptor-associated factor 3	1.72
TUBB6	209191_at	tubulin, beta 6	1.61
VCAN	215646_s_at	versican	2.50
VCL	200931_s_at	vinculin	2.17