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Enhanced expression of SOS1 is detected in prostate cancer epithelial cells from African American men

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Abstract

African-American (AA) men experience increased risk of developing prostate cancers as well as increased mortality following treatment as compared to European-American (EA) men. The aim of our study was to identify biological factors with potential to predispose AA men to prostate tumor progression and metastasis. To identify cancer-specific gene expression patterns in AA men, we established primary prostate cancer epithelial cells from 14 AA and 13 EA men. High-throughput microarrays were used to investigate differences in global gene expression comparing the two groups. Quantitative RT-PCR and immunohistochemistry validated mRNA and protein expression levels. RNAi knockdowns provided support for biological significance for the identified genes in prostate cancer cells. Son of sevenless homolog 1 (SOS1) was overexpressed in AA-derived primary prostate cancer epithelial cells. Depletion of SOS1 in PC3 and DU145 prostate cancer cells resulted in decreased capacities for cell proliferation, migration and invasion, at least partially through inhibition of extracellular signal-regulated kinase 1 and 2 (ERK1/2). Tissue microarray analyses of SOS1 expression in prostate carcinomas correlated with Gleason's grades of tumors, consistent with a possible role in prostate cancer progression. Investigation of prostate cancer derived epithelial cells has led to identification of SOS1 as a potential candidate biomarker and molecular therapeutic target in prostate cancer in AA men, consistent with the hypothesis that a biological basis exists for prostate cancer aggressiveness in AA men.

Keywords

Prostate cancer; African American men; global mRNA expression profiling; migration and survival

Introduction

Prostate cancer is the most frequently diagnosed solid malignancy in American men, and results in approximately 30,000 deaths annually (1). Comparisons of population-based registries consistently show that African American (AA) men have the highest age-adjusted incidence (2). In the period 1996–2000, AA men had an age-adjusted incidence 1.6 times higher that of European American (EA) men (3). In addition, AA men have worse overall survival (risk ratio 1.35, 95% CI 1.23–1.48) (4). The reasons for elevated incidence and

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poorer prognosis in AA men are not clear. Differences in access to health care and management of local disease, and differences in disease biology have been suggested (5). However, recent studies have adjusted for socioeconomic variables, but continued to find increased risk among AA men for prostate cancer specific survival and biochemical recurrences (4–9). Therefore, examination of potential biological differences offers an approach to identify and reduce this health disparity.

Previous investigations have identified genetic factors with potential for increasing prostate cancer incidence and mortality in AA, suggesting differences in tumor biology (10–13). Such studies have addressed differences in the distributions of known or suspected risk factors among racial groups. Efforts to correlate more aggressive biology with racial differences have included whole-genome prostate cancer association studies which have identified susceptibility loci for prostate cancer (14–16). Increased androgen levels and androgen receptor expression have been implicated in the development and rapid progression of prostate cancer in AA (17–19). Also, higher levels of insulin-like growth factor-I and lower levels of IGF binding protein-3 have been associated with an increased risk of prostate cancer (20). However, such studies have not identified the presumed cancer-relevant genes and the reasons for increased prostate cancer risk in the AA population remain to be defined.

Three reported studies have used gene expression profiling to compare prostate tissue from AA and EA patients (21–23). The study by Haqq et al. compared gene expression in non-neoplastic prostate tissues from various ethnic groups (21). No cluster node distinguished non-cancerous prostate tissue of AA from EA men, although differential expression levels of estrogen receptor alpha (ERa) were observed in the stroma (21). The study by Wallace et al. analyzed differences in gene expression in prostate tumors from 33 African American and 36 European American men (22). This analysis revealed higher expression of genes that influence immune responses and metastasis in the tumors of African-American men compared with European-American men. Among the genes showing elevated expression in prostate tumors from African-American men were AMFR, CXCR4, and MMP9, all of which have been linked to cancer metastasis (22). The study mostly detected differential expression of immune responsive genetic programs in AA as compared to EA patients that offer potentially important leads for understanding the disease (22). The most recent study by Reams et al. compared gene expression profiling in tumors with a Gleason score of 6 from African-American males to prostate tumors in European American males (23). This study also showed that the gene ontology terms prevalent in African American prostate tumor/normal ratios included interleukins, progesterone signaling, chromatin-mediated maintenance and myeloid dendritic cell proliferation (23).

The high degree of heterogeneity of prostate tissue presents a challenge for molecular studies of prostate cancer. The analysis of tumor tissues offers insight into contributions by both, epithelial and stomal components; however, the presence of fibroblasts, inflammatory cells, and vascular endothelial cells, increase the complexity in interpreting expressed gene patterns and must be taken into account when performing high-throughput analyses. Moreover, representation of each cell type within a given sample determines the overall expression profile and makes it difficult to compare prostate samples with varying epithelial and stromal contents (24). In this study we established primary cell strains from radical prostatectomy specimens of AA and EA men, which were matched for tumor stage and Gleason's grade. Global gene expression analyses in these primary epithelial cells showed increased levels of expression of genes associated with growth, migration and invasion in AA as compared to EA men suggesting a difference in epithelial cancer cell biology. Our examination of the roles of these genes in modifying cellular characteristics demonstrated

enhanced growth and migration of prostate cancer cells, consistent with a more “aggressive” phenotype.

Materials and methods

Generation of primary cell culture

The malignant tissues from African American and European American men used for generating primary cultures were obtained from radical prostatectomy specimens according to Walter Reed Medical Center and Uniformed Services University of the Health Sciences Internal Review Board approved protocols. The presence of prostatic adenocarcinoma was confirmed by light microscopy. Fresh prostatectomy tissue specimens were obtained under sterile conditions by an experienced pathologist. Tumor tissue on gross inspection was dissected separately for the purpose of generating a cell culture. The method for generating primary cell cultures has been previously described (25, 26). Briefly, minced pieces of tissues were distributed to several collagen coated cell culture dishes with keratinocyte-serum free medium (K-SFM) (Life Technologies. Inc. Gaithersburg, MD) supplemented with 5% fetal bovine serum. The serum was used to stimulate primary prostate epithelial cells in culture (27). Tissue explants were grown for three weeks until reaching confluence. Aliquots of the primary cultures were then frozen and stored in liquid nitrogen until the cells were reestablished in secondary culture.

Cell growth and maintenance

Reconstituted primary cells were cultured for less than 5 passages. Cells were grown to the same confluence (about 80%) in a K-SFM supplemented with bovine pituitary extract and recombinant epidermal growth factor (Life Technologies, Inc., Gaithersburg, MD). Cells from twenty eight patients were expanded and 27 (14 AA and 13 EA) yielded sufficient cell numbers for gene expression studies.

Affymetrix microarray analysis

Total RNA was extracted using an RNeasy kit (Qiagen, Valencia, CA, USA). RNA labeling and hybridization were performed according to Affymetrix standard protocol for one-cycle target labeling method. Fragmented cRNA was hybridized in triplicates to Affymetrix GeneChip HG-U133A 2.0 arrays (Affymetrix, Santa Clara, CA).

Data analysis, bioinformatics, and statistics

Affymetrix microarray data were analyzed to identify differentially expressed genes in the epithelial cells from the cohorts of AA (n=14) and EA (n=13) prostate cancer patients. Affymetrix data analysis included pre-processing of the probe-level Affymetrix data (CEL files). We applied RMA for background adjustment, quantile method for normalization, and the “median polish” for summarization. The triplicate arrays representing the same subject were averaged. The random variance model implemented in BRB-ArrayTools (NCI, Bethesda, MD) was used for this analysis (28). Probe sets were considered statistically significant if their p-values were less than 0.001. For each gene, BRB-ArrayTools calculated the False Discovery Rate (FDR), which is defined as the median number of false positive genes divided by the number of significant genes. We have also used BRB-ArrayTools to determine the most enriched binding sites in the promoters of differentially expressed genes. Pathway analysis was performed with Database for Annotation, Visualization and Integrated Discovery (DAVID) (29).

Quantitative RT-PCR

Total RNA extracted from primary cell strains (8 strains for each group) was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR (qRT-PCR) was performed in triplicate using TaqMan Gene Expression Assays (Applied Biosystems) on the Applied Biosystems 7900HT Fast Real-time PCR System using standard mode. The assay ID numbers of the validated genes were as follows: autocrine mobility factor receptor (AMFR), Hs00181609_m1; cathepsin B (CTSB), Hs00947439_m1; catenin (cadherin-associated protein), beta 1, 88kDa (CTNNB1), Hs00170025_m1; son of sevenless homolog 1 (SOS1), Hs00362308_m1; metastasis associated 1 family, member 2 (MTA2), Hs00191018_m1; homeodomain interacting protein kinase 3 (HIPK3), Hs00178628_m1; cyclin D2 (CCND2), Hs00277041_m1. Amplification of 18S rRNA was used as an endogenous control to standardize the amount of sample added to the reaction. The comparative cycle threshold (CT) method was used to analyze the data by generating relative values of the amount of target cDNA (Applied Biosystems). CT represents the number of cycles for the amplification of target to reach a fixed threshold and correlates with the amount of starting material present. To obtain relative values, the following arithmetic formula was used: $2^{-\Delta\Delta CT}$, where ΔCT = difference between the threshold cycles of the target and an endogenous reference (18S), and $-\Delta\Delta CT$ = difference between ΔCT of the target sample (AA) and a designated calibrator (EA). The statistical analyses of these data were performed with a two-sided *t* test if the expression data showed normal distribution; otherwise the Wilcoxon Rank-Sum test was applied.

Immunohistochemistry

AMFR, SOS1, and MTA2 protein distributions in prostate tissues were determined by immunohistochemical staining of two normal and three tumor prostate tissues. Five micron sections from formalin fixed paraffin embedded tissues were deparaffinized with xylenes and rehydrated through a graded alcohol series. Antigen retrieval was performed by immersing the tissue sections at 98° C for 20 minutes in 10 mM citrate buffer (pH 6.0) with 0.05% Tween. Immunohistochemical staining was performed using the Histostain Plus kit from Zymed according to manufacter's instructions. Briefly, slides were exposed to 1/50, 1/100, and 1/100 dilution of primary antibodies for AMFR, SOS1, and MTA2 (Santa Cruz Biotechnology, Santa Cruz, CA) respectively for 1 hour at room temperature (RT) and biotin-conjugated broad spectrum secondary antibodies were applied. Horseradish peroxidase conjugated streptavidin was added for 10 minutes at RT and HRP detected by DAB (Dako). Slides were counterstained with Hematoxilyn (Fisher, Harris Modified Hematoxilyn) at a 1:17 dilution for 2 minutes at RT, blued in 1% ammonium hydroxide for 1 minute at RT, dehydrated, and mounted with Permount. Consecutive tumor sections with the omitted primary antibody were used as negative controls. Images were captured using an Olympus DP70 microscope at 40x magnification.

Prostate cancer TMAs containing 33 cases/63 cores (BC19012) were purchased from US Biomax, Rockville, MD and processed according to manufacturer's recommendations. Three TMA slides were stained with each antibody. The intensity and extent of the immunoreactivity were microscopically evaluated in each core on three slides by a pathologist (B.VS.K.) experienced in diagnosing prostate carcinoma. Staining intensity was scored on an arbitrary 0 to 3+ scale, with 0 indicating no or trace staining and grades 1+ to 3+ representing increasing intensity. The extent of positive intracytoplasmic staining was evaluated in a semiquantitative manner. Scoring was based on percentage of stained epithelial cells and graded from 0 to 3, signifying 0–4%, 5–25%, 26–50% and >50%, respectively.

siRNA knockdown

Stealth RNAi (RNAi) to silence the expression of AMFR or SOS1 and non-specific Block-it RNA were obtained from Invitrogen (Carlsbad, CA). The controls included non-silencing Cy3-labeled siRNA and Cy3-labeled GAPDH siRNA, obtained from Qiagen (Austin, TX). PC3 cells were transfected with 10–50 nM siRNA. Transfection was performed using Trans-TKO reagent from Mirus (Madison, WI) according to the manufacturer's instructions. Total RNA and protein were extracted 24, 48, 72, and 96 hrs and 7 days after transfection. The SOS1 and AMFR mRNA levels were measured by qRT-PCR using TaqMan Gene Expression Assays (Applied Biosystems) on the Applied Biosystems 7900HT Fast Real-time PCR System using standard mode as described above. Western blots were performed with anti-AMFR and anti-SOS1 antibody from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Proliferation and FACS Analysis of Cell Cycle—PC3 and DU145 cells were seeded at 0.3×10^5 per well in six-well plates and maintained in RPMI medium supplemented with 10% FBS for 24 h before being transfected with RNAi. Two days after the RNAi treatments, cells were re-plated at 0.2×10^5 per well in six-well plates. Cell proliferation rates were measured by cell counting of samples in triplicate every 2 days. For flow cytometry, cells were cultured for 72 h after RNAi transfection, fixed in 70% ethanol, stained with propidium iodide, and analyzed for cell cycle distribution by FACScan as previously described (30).

Wound healing assay

Cells were allowed to grow to complete confluence, and then starved in low serum (0.5%) for 24 hrs. Subsequently, a plastic pipette tip was used to scratch the cell monolayer to create a cleared area, and the wounded cell layer was washed with fresh medium to remove loose cells. Immediately following scratch wounding (0 h) and after incubation of cells at 37 °C for 20 h, phase-contrast images (10xfield) of the wound healing process were photographed digitally with an inverted microscope (Olympus IX50). The distances of the wound areas were measured on the images, set at 100% for 0 h, and the mean percentages of the total distances of the wound areas were calculated.

Invasion assay

DU145 and PC3 cells were transfected with SOS1 RNAi, AMFR RNAi and control RNAi and seeded in duplicates at 5×10^4/insert in BD BioCoat™ 24-Multiwell Tumor Invasion System (BD Biosciences) according to manufacturer's recommendations. After 20-hrs incubation, inserts were transferred to 24-well plates containing fluorescent dye Calcein AM (Molecular Probes) at 4mg/ml in Hank's Balance Salt Solution and incubated in 37°C incubator at 5% CO2 for 1.5 hours. Plates were read at excitation 485 nm/emission 530 nm in Applied Biosystems CytoFluor® 4000 multi-well plate reader.

Results

Survival, migration and invasion genes are upregulated in prostate cancer epithelial cells from African American men

To investigate potential biological differences in epithelial cells in ethnic populations, we established primary cell cultures from prostate tumors resected from AA and EA men as described (25, 26). Cultured cells, showing typical epithelial cell morphology were selected for gene expression profiling (14 AA and 13 EA cell cultures). Table 1 shows matching of the two cohorts of patients by age, tumor stages, Gleason's score, seminal vesicles and lymph node involvement. Microarray data analysis identified 382 differentially expressed genes satisfying the selection criteria of p-value of 0.001 or less and False Discovery Rate

(FDR) of 0.005 (0.5%) or less (data will be deposited in GEO database). Supervised hierarchical cluster analysis showed that 67 genes were increased more than 1.5-fold in AA and 25 genes were increased more than 1.5-fold in EA and partitioned two ethnic groups as demonstrated in the heatmap (Figure 1). qRT-PCR for selected genes, such as: autocrine motility factor receptor (AMFR), metastasis-associated 1 family, member 2 (MTA2), son of sevenless 1 (SOS1), cyclin D2 (CCND2), cathepsin B (CTSB) and homeodomain-interacting protein kinase 3 (HIPK3), confirmed their higher expression levels in prostate cancer epithelial cells from AA men (Figure 2). In addition, immunohistochemical analyses of normal prostate tissues and high grade prostate carcinomas demonstrated that genes identified in our study, such as AMFR, SOS1 and MTA2, are primarily expressed in epithelial cells in prostate tissues (Figure 3). These data confirm that cells isolated from prostatectomy specimens were enriched for the genes differentially expressed in the epithelial component of prostate tumors.

To determine biological pathways significantly overrepresented in prostate cancer epithelial cells from AA men, the 92 differentially expressed genes with 1.5 fold cut off were analyzed using DAVID software (29). The identified biological processes include signal transduction, cell communication, focal adhesion, cell cycle and apoptosis (Table 2). These same pathways were overrepresented in the study reporting comparisons of tumor tissues (22). While the tumor tissue analysis demonstrated overrepresentation of immune response processes, these biological processes were absent in our study consistent with our focus on gene expression in epithelial cancer cells. Closer examination of the expression data revealed that tumor cells from AA show increases of expression of genes involved in survival, proliferation, migration, invasion, and metastases (Table 3). We found that 13 genes upregulated and 2 genes downregulated in prostate cancer of AA men were common between Wallace's and our studies (Table 4). Among genes upregulated in both studies were *PSPHL* (probe set 205048_s_at) and *CRYBB2* (probe set 206777_s_at). According to Wallace et al., this two-gene signature accurately differentiates between tumor tissues from African-American and European-American patients, but not between non-tumorous tissues providing additional validation for our system. Notably, the use of cell cultures allowed determination of differential expression of common genes with higher confidence (Table 4). This suggests that some genes identified in our study may have been missed in analyses of tumor tissues.

KEGG pathway database analyses of differentially expressed genes in the revealed that 7 upregulated genes are closely associated with the MAPKK cascade, deregulated in the epithelial cells from prostate cancers of African American men (Table 2). Recently, aberrant MAPK pathway has emerged as one of the key events in the multistep nature of prostate tumorigenesis and progression (31, 32). An upstream activator of MAPK signaling pathway, SOS1, is 2-fold upregulated in prostate cancer epithelial cells from AA men (Figure 2). These data provided a rationale for further studies of the roles of SOS1 in prostate cancer progression, in particular in African American men.

SOS1 increase proliferation, migration and invasion in established model human prostate cancer cells

To determine how SOS1 may affect critical characteristics of prostate cancer cell behavior we knocked down levels of SOS1 in prostate cancer DU145 and PC3 cells (Figure 4A) and measured proliferation, migration, and invasion. The choice of cell lines was dictated by the lack of prostate cancer epithelial cell lines derived from African American patients and aggressive phenotype of both PC3 and DU145 cells (33). Decreases in SOS1 levels inhibited growth of both, PC3 and DU145 prostate cancer cells (Figure 4B). Cell cycle analysis showed that knockdown of SOS1 causes G0/G1 phase cell cycle arrest and triggers cell death as suggested by increased numbers of cells in the sub-G1 phase (Figure 4C). The

wound healing and BD BioCoat™ Tumor Invasion assays demonstrated that decreases in SOS1 levels inhibit migration and invasion of prostate cancer cells (Figure 4D, E). Decreases of SOS1 levels diminished levels of phospho-ERK1/2. In addition, SOS1 siRNA downregulated levels of EGFR in DU145 prostate cancer cells (Figure 5). These events may contribute to decreased survival and migration of prostate cancer cells, since the Ras-MEK-ERK and EGFR signaling pathways can sustain growth, migration and invasion (33, 34). Therefore, increases in the levels of these proteins may contribute to prostate cancer aggressiveness in African American men.

Elevated SOS1 expression correlates with high Gleason's sum

Gleason's sum of tumor tissues has been shown to correlate to clinical aggressiveness of prostate cancers. We reasoned that gene products contributing to prostate cancer aggressiveness should also show differential presence in tissues with higher Gleason's sum. To determine how expression of SOS1 correlates with prostate cancer aggressiveness, we analyzed protein expression in a prostate carcinoma tissue microarray containing 30 samples of prostate carcinoma with various Gleason's sum and 3 samples of benign tissue. Moderate and strong expression levels of SOS1 protein was observed in tissues with Gleason's sum ≥7, whereas benign tissues and prostate tumors with Gleason's sum 6 showed only weak expression of SOS1 (Table 5). There was no correlation between Gleason's sum and the number of cells expressing SOS1. We interpret this finding to support a role for SOS1 in prostate cancer aggressiveness and suggest SOS1 as a potential biomarker suitable for further evaluation in prostate cancers from AA men.

Discussion

We have analyzed primary prostate cancer cells in culture to identify differentially expressed genes in the epithelial component of cancers in AA and EA men. This approach allows simplification of epithelial cell analyses, free of interactions with stroma and inflammatory cells, unavoidable in studies of prostate cancer tissues. Primary cultures are widely used to investigate the disease-specific biology of prostate cancer and benign prostatic hyperplasia (35). Study of gene expression profiles using Affymetrix Human Cancer G110 Array Chips containing approximately 1900 cancer-related genes did not show consistent over-expression of any gene in cancer cells compared to normal prostate epithelial cells (35). Using genome-wide expression profiling we demonstrate that 17 genes related to processes associated with growth, migration, invasion, and metastases, including AMFR, SOS1, MTA2, CTSB, CTNNB1, and AXL are upregulated in AA epithelial cells. In addition, 10 genes drive cell cycle progression, including cyclin D1 and D2, HIPK3 and JUN. Some of these genes were detected previously in the study by Wallace et al., but with lower confidence based on p-value and false discovery rate (22). Immunohistological staining demonstrated expression of AMFR, SOS1 and MTA2 in prostate cancer epithelial cells and not in stromal cells. Taken together, our data suggest that studies of gene expression in epithelial cell cultures offer greater sensitivity than prostate tumor tissue in identifying differences specific to malignant epithelial cells.

We observed that SOS1, activator of Ras/mitogen-activated protein kinase (MAPK), is upregulated in AA prostate cancer cells. This may underlie MAPK signaling cascade deregulation in AA prostate cancer cells, as predicted by the bioinformatics analysis of microarray data. The Ras/MAPK signaling cascade represents a pivotal molecular circuitry for prostate cancer evolution, driving cell proliferation and enhancing cell migration, invasion and metastases (32, 36, 37). It has been previously shown that inhibition of ERK signaling with small molecule inhibitors or siRNA inhibited prostate cancer cell proliferation (34), grossly impaired clonogenicity and invasion through Matrigel (33). As an activator of Ras and hence MAPK signaling, SOS1 may be a promising cancer therapy

target. Here we have demonstrated that SOS1 siRNA knockdown decreases cell survival and proliferation, migration and invasion in prostate cancer cells. The mechanism for such an inhibition, at least partially, relies on inhibition of ERK1/2 phosphorylation. We propose that SOS1 increased levels may contribute to prostate cancer cell proliferation and migration through activation of ERK signaling. The tissue microarray data suggest that increased expression of SOS1 correlates with higher Gleason's score, and hence with prostate cancer aggressiveness. Further correlation of SOS1 expression and ERK1/2 activation in prostate carcinomas from AA men is underway to understand the molecular mechanism by which SOS1 promotes cancer progression in AA men and establish the paradigm for anticancer drug development.

In addition, we have detected enhanced expression of AMFR in prostate cancers from AA, which was previously detected in the study by Wallace et al. (22). The biological functions of AMFR relate to migration and metastases. Stimulation of AMFR by its ligand autocrine motility factor alters cellular adhesion, proliferation, motility, and apoptosis. AMFR upregulation correlates with more advanced tumor stage and decreased survival for cancers of the lung, esophagus, stomach, colon, rectum, liver and skin (38). AMFR also serves as an independent predictor of poor disease prognosis in these various tumor types (38). We found that decrease in AMFR levels with siRNA induces death in prostate cancer cells and inhibits their migration (data not shown). Downstream signaling mechanisms activated by AMFR are not completely understood. Recently, a role for AMFR in sarcoma metastasis has been suggested through targeting of the transmembrane matastasis suppressor, KAI1, for degradation (39). Notably, KAI1 protein expression is downregulated in more than 70% of primary prostate cancers, and more than 90% of metastatic prostate cancers (40, 41). This suggests the possibility that upregulation of AMFR in prostate cancers impacts degradation of KAI1 to contribute to disease progression through increased survival and migration. Further studies are necessary to establish a role of AMFR in prostate cancer and to validate it as a molecular target for anticancer therapy.

In summary, we have investigated gene expression in prostate cancer to address racial health disparity. That AA men present with more advanced prostate cancers at diagnosis, and demonstrate more aggressive disease progression than do EA men has been supported by epidemiological studies (4, 16). Health disparity investigations have generally focused on access to health care and socioeconomic status, however, disparity in prognosis is observed even for patients participating in health maintenance organizations, in which access to health care is comparable for AA and EA patients (16). African American males whose prostate surgical margins contain cancer cells seem to have greater biological aggressiveness of residual disease, a higher clinical recurrence rate of disease, and a lower survival rates even after radical prostatectomy (42). Our approach does not include epithelial-stromal or -inflammatory cells interactions, which have been shown to contribute to the carcinogenic process (43). However, our approach does offer to provide insight into the prostate cancer epithelial cells, the presumed vehicle of the malignant process. Our data identify SOS1 and AMFR as representative biological factors of cancer aggressiveness consistent with the clinical findings of increased stages at diagnosis and more aggressive clinical courses of prostate cancer in AA as compared to EA patients. Confirmation of these molecules in a larger, prospective study is needed to validate the candidate genes as biomarkers or therapeutic targets.

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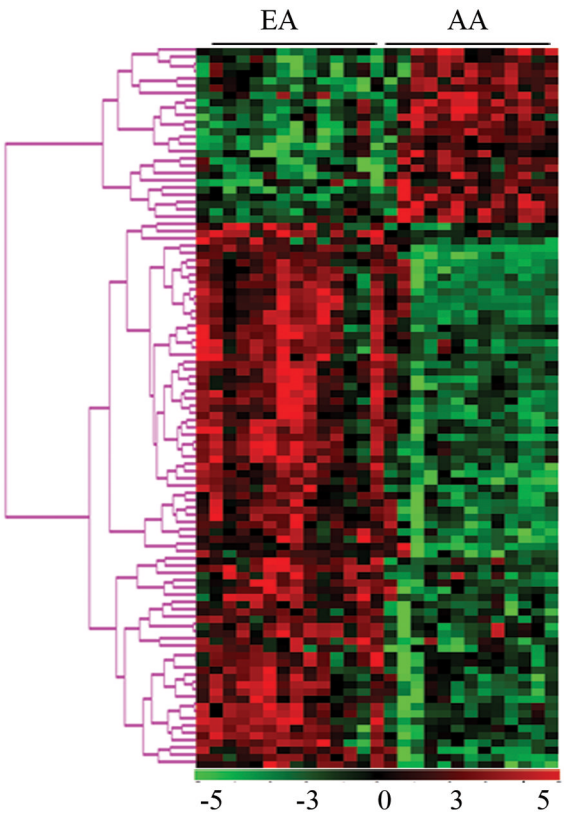

Figure 1. Heatmap of up- and down-regulated genes with fold change >1.5 in AA and EA

Genes are ordered according to their cluster determined by the *k*-means algorithm. The first 14 cell strains are from AA men, the other 13 cell strains are from EA men. Green represents lower expression and red represents higher expression.

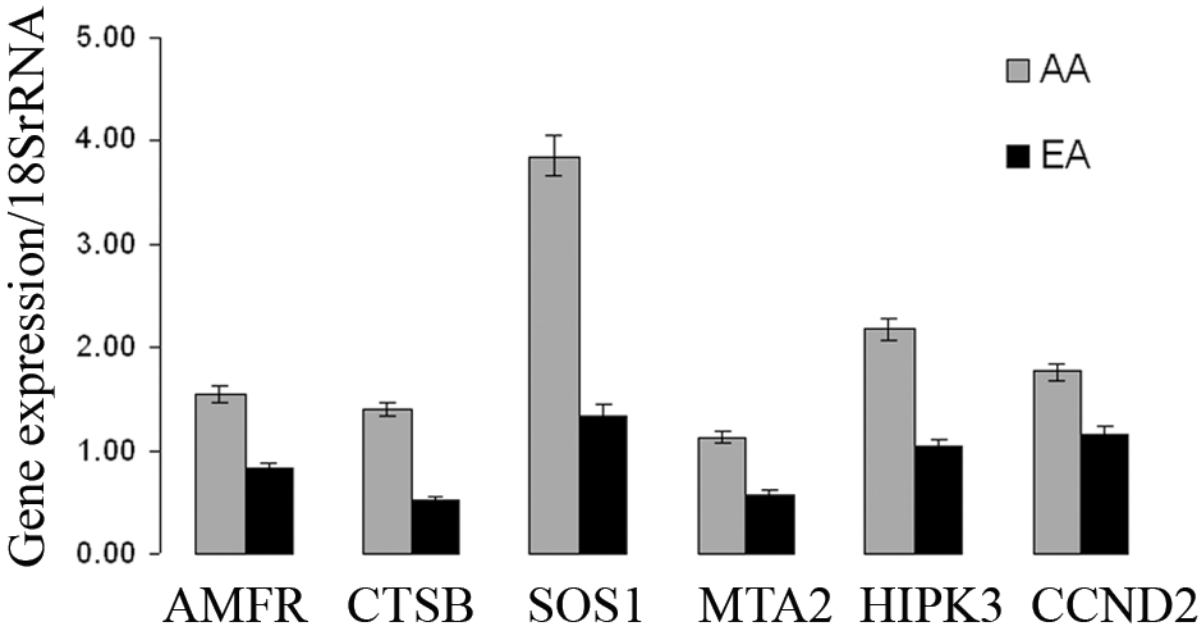

Figure 2. Differential gene expression in prostate epithelial cells from African American and European American men

qRT-PCR expression analysis of AMFR, SOS1, MTA2, HIPK3, cathepsin B, and cyclin D2 normalized by 18SrRNA. Each bar represents a mean of three independent experiments for one cell line; bars represent SD.

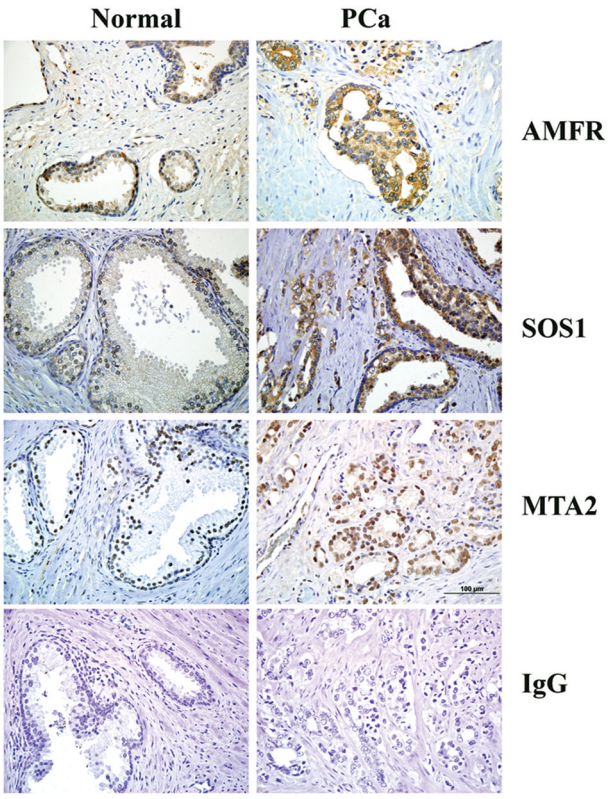

Figure 3. AMFR, SOS1, and MTA2 are over-expressed in clinical prostate cancer tissue specimens

Paraffin embedded tissue sections of human normal prostate and prostate cancer specimens were immunostained with a polyclonal antibody against human AMFR, SOS1, and MTA2. Diaminobenzidine tetrachloride (DAB) and Mayers hematoxylene were used as chromogenic substrates and counter stains respectively. Intense AMFR, SOS1, MTA2 immunoreactivity is seen in epithelial cells. AMFR and SOS1 immunostaining were observed in the cytoplasm, whereas MTA2 immunostaining was detected in the nuclei (original magnification x40). Subtype specific pure rabbit IgG was used as a negative control.

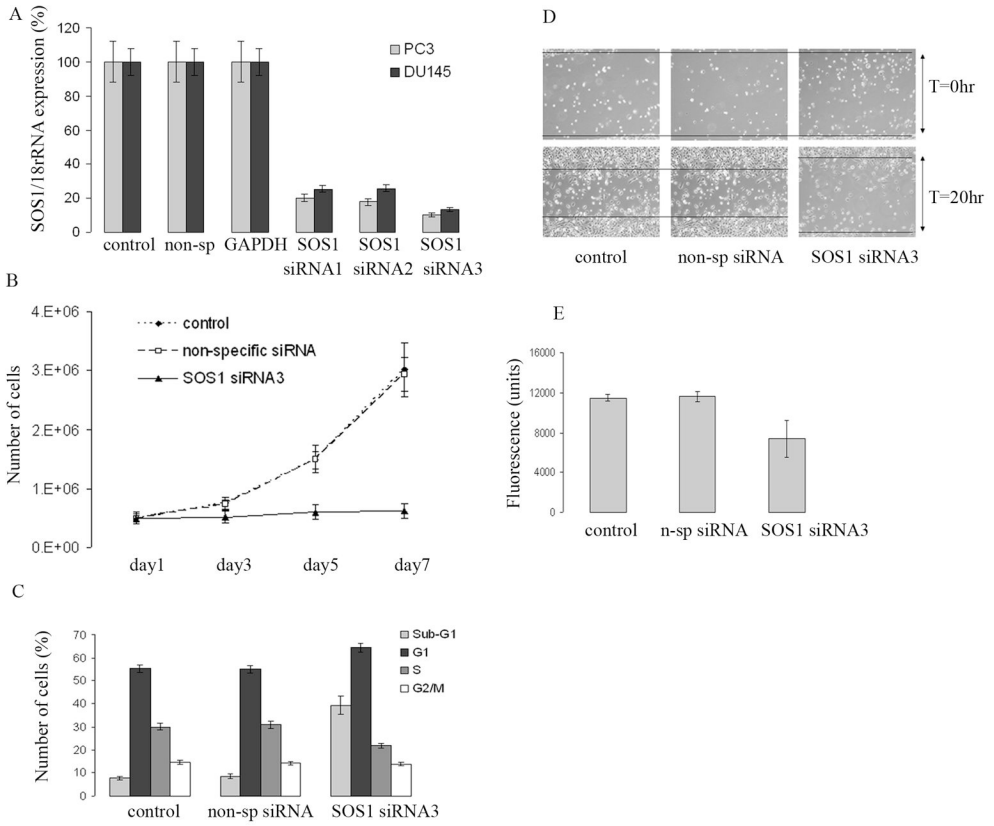

Figure 4. Decrease in SOS1 levels causes inhibition of prostate cancer cell proliferation, survival, and invasion

A) Levels of SOS1 were downregulated using three different stealth siRNAs. SOS1 mRNA levels in PC3 and DU145 prostate cancer cell lines were measured by qRT-PCR 24 hours after transfection with RNAi and normalized against 18S rRNA levels. B) Cell growth curves were obtained as follows: two days after transfection 500,000 cells were plated in 100 mm dishes in triplicates; cells were trypsinized and counted every 2 days. The graph is shown for DU145 cells. C) Cell cycle analyses were performed on DU145 transfected cells fixed with EtOH 5 days after transfection. D) Cells were plated in 6-well plates and transfected with non-specific siRNA or SOS1 siRNA3 in triplicates (controls cells were left untreated). After cell cultures reached 100% confluence, cells were starved for 24 hours and the scratched has been made. Photographs were taken immediately (T=0 hr) and 20 hours after making a scratch (T=20hr). E) Invasion assays were performed two days after transfection. DU145 cells were plated in 24-well FluoroBlock plates in duplicates and after 20 hr stained with calcein AM. Fluorescence readings of cells migrating through the pores to the lower chamber were taken with a plate reader at 485 nm excitation and 530 nm emission.

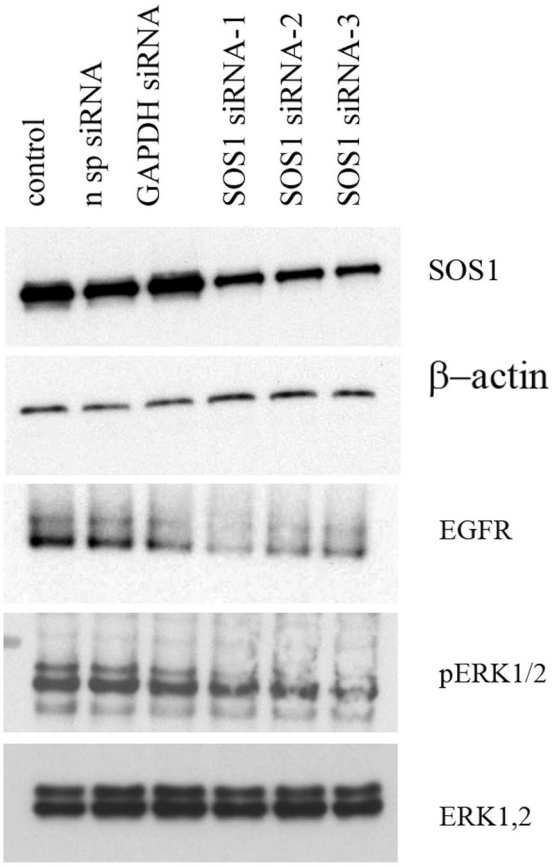

Figure 5. SOS1 RNAi knockdown decreases levels of pERK and EGFR in prostate cancer DU145 cells

Levels of pERK and EGFR were measured 48 hours after transfection with non-specific scrambled siRNA (n sp siRNA), GAPDH siRNA, and SOS1 siRNA 1, 2 and 3, in the whole DU145 cell protein extracts. The membranes were re-blotted with antibody against b-actin and ERK for loading control, and with antibody against SOS1 to demonstrated knockdown efficiency.

Table 1

Clinical Characteristics of the study population

Categories	All cases (*n* = 27)	African- American (*n* = 14)	European-American (*n* = 13)	*P** (*t* test)
Age at prostatectomy	60.4 (42.2–75.6)	61.4 (46.1–71.6)	60.2 (42.2–75.6)	0.708
	N	N	N	P (Fisher's exact test)
Pathologic stage				
pT2, *n* (%)	14	7	7	0.712
≥pT3, *n* (%)†	12	7	5	
Gleason's sum				
<7 (5–6), *n* (%)	10	4	6	0.440
≥7 (7–9), *n* (%)	17	10	7	
Seminal vesicle invasion†				
No, *n* (%)	23	11	12	0.225
Yes, *n* (%)	3	3	0	
Nodes invasion†				
No, *n* (%)	16	11	5	0.530
Yes, *n* (%)	3	3	0	
Surgical margin status†				
Negative, *n* (%)	17	7	10	0.110
Positive, *n* (%)	9	7	2	

* *P* value for the difference between African-Americans and European-Americans. All tests were two-sided.

† Cases with unknown status are not included

Table 2

Statistically significantly (p-value < 0.05) altered gene ontology biological process terms in AA-altered genes

GOTERM/ KEGG	Term hits (of 92)	P-value	Enrichment score
Signal transduction	27	4.2E-2	2
Cell communication	30	2.4E-2	2
Apoptosis	8	9.8E-3	0.73
Cell cycle	10	3.4E-2	2
Focal adhesion	8	2.4E-4	2.04
Prostate cancer	5	2.4E-3	2.04
MAPK signaling	7	7.0E-4	1.99
Wnt signaling	7	2.3E-6	2.07

Table 3

Differential Gene Expression – Metastasis, Migration and Invasion Related

Gene symbol	Description	FC (AA vs. EA)	Parametric p-value
AMFR	autocrine motility factor receptor	4.2	<1e-07
CTSB	cathepsin B	2.5	0.0001552
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	1.9	0.0000002
SOS1	son of sevenless homolog 1 (Drosophila)	1.9	<1e-07
MTA2	metastasis associated 1 family, member 2	1.6	0.0000023
AXL	AXL receptor tyrosine kinase	1.6	0.0006603
PLA2G4C	phospholipase A2, group IVC (cytosolic, calcium- independent)	1.7	0.0008874
ARPC4	actin related protein 2/3 complex, subunit 4, 20kDa	1.6	0.0000138
PTP4A1	protein tyrosine phosphatase type IVA, member 1	1.6	9.07E-05
PPFIBP1	PTPRF interacting protein, binding protein 1 (liprin beta 1)	1.6	3.92E-05
MARCKS	myristoylated alanine-rich protein kinase C substrate	1.6	2.38E-05
JUN	jun oncogene	1.6	2.30E-06
PKN2	protein kinase N2	1.6	1.50E-06
LAMP1	lysosomal-associated membrane protein 1	1.5	0.0000271
CTTN	cortactin	1.5	0.0000111
CRKL	v-crk sarcoma virus CT10 oncogene homolog (avian)-like	1.5	0.0000159
PKP1	plakophilin 1 (ectodermal dysplasia/skin fragility syndrome)	1.5	0.0001119

Table 4

Genes detected in two independent studies

Gene symbol	*Description*	*Our study*			*Wallace et al., 2008*		
		FC	**p-value**	**FDR**	**FC**	**p-value**	**FDR**
AMFR	autocrine motility factor receptor	4.2	1.0E-07	0	1.9	2.4E-05	0
PSPH	phosphoserine phosphatase	3.4	1.0E-07	0	4.6	1.0E-11	0
CRYBB2	crystallin, beta B2	2.4	1.0E-07	0	3.0	1.3E-11	0
SOS1	son of sevenless homolog 1 (Drosophila)	1.9	1.0E-07	0	1.4	1.3E-03	2
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	1.9	1.0E-07	0	1.3	4.1E-07	0
CCND1	cyclin D1	1.5	1.0E-07	0	1.2	3.0E-02	16
HCLS1	hematopoietic cell-specific Lyn substrate 1	1.5	2.9E-05	0	1.4	7.9E-03	6
SLC7A5	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	1.5	8.2E-04	0	1.3	3.0E-02	16
ARTS-1	type 1 tumor necrosis factor receptor shedding aminopeptidase regulator	1.5	2.9E-05	0	1.3	3.8E-03	5
SOCS1	suppressor of cytokine signaling 1	1.5	2.9E-05	0	1.3	1.0E-02	10
ISG20	interferon stimulated exonuclease gene 20kDa	1.4	5.5E-04	0	1.3	8.2E-03	6
CROCCL1	ciliary rootlet coiled-coil, rootletin-like 1	1.3	1.6E-06	0	1.3	7.2E-03	6
FLI1	Friend leukemia virus integration 1	1.3	2.7E-04	0	1.3	2.0E-02	13
RPL47A	ribosomal protein L37a	0.6	1.0E-07	0	0.8	1.0E-02	20
ADI1	acireductone dioxygenase 1	0.5	1.0E-07	0	0.7	1.2E-04	0

Table 5

Expression of SOS1 in prostate carcinomas with different Gleason's score

Gleason's sum	# samples	negative	weak +	moderate/strong ++/+++
benign	3	2	1	
<7	7		7	
≥7	23	3	11	9