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Identifying and treating *ROBO1*$^{-ve}$/*DOCK1*$^{+ve}$ prostate cancer: An aggressive cancer sub-type prevalent in African-American patients

Marina G. Ferrari, BS[1], **Arsheed A. Ganaie, PhD**[1], **Ashraf Shabenah, MS**[2], **Adrian P. Mansini, PhD**[1], **Li Wang, PhD**[3], **Paari Murugan, MD**[4], **Elai Davicioni, PhD**[5], **Jinhua Wang, PhD**[2], **Yibin Deng, MD PhD**[1], **Luke H. Hoeppner, PhD**[3], **Christopher A. Warlick, MD**[1], **Badrinath R. Konety, MD**[1], **Mohammad Saleem, PhD**[1]

[1]Department of Urology, School of Medicine, Masonic Cancer Center, University of Minnesota

[2]Institute for Health Informatics, Masonic Cancer Center, University of Minnesota

[3]Hormel Institute, University of Minnesota, Austin, MN

[4]Department of Laboratory Medicine and Pathology, University of Minnesota,

[5]Decipher Biosciences, Inc. San Diego, CA

Abstract

Background: There is a need to develop novel therapies which could be beneficial to prostate cancer (CaP) patients including those who are predisposed to poor outcome such as African-Americans. This study investigates the role of *ROBO1*-pathway in predicting outcome and race-based disparity in CaP patients.

Methods and Results: Aided by RNA sequencing-based DECIPHER-testing and IHC analysis of tumors we show that *ROBO1* is lost during the progressive stages of CaP, a prevalent feature in African-Americans. We show that the loss of *ROBO1* predicts high-risk of recurrence, metastasis and poor outcome of ADT in RP-treated patients. These data identified an aggressive *ROBO1*^{deficient}/*DOCK1*$^{+ve}$ sub-class of CaP. Combined genetic and IHC data showed that *ROBO1* loss is accompanied by *DOCK1*/*Rac1* elevation in Grade-III/IV primary-tumors and Mets. We observed that the hypermethylation of *ROBO1*-promoter contributes to loss of expression that is highly prevalent in African-Americans. Because of limitations in restoring ROBO1 function, we asked if targeting the DOCK1 could be an ideal strategy to inhibit progression or treat *ROBO1*^{deficient} metastatic-CaP. We tested the pharmacological efficacy of CPYPP, a selective inhibitor of DOCK1 under *in vitro* and *in vivo* conditions. Using *ROBO1*$^{-ve}$ and *ROBO1*$^{+ve}$ CaP models, we determined the EC_{50} for growth. DOCK1-inhibitor treatment significantly decreased the (i) Rac1-GTP/β-catenin activity, (ii) transmigration of *ROBO1*^{deficient} cells across endothelial lining and (iii) metastatic spread of *ROBO1*^{deficient} cells through the vasculature of transgenic^{fl} Zebrafish model.

[*]**Correspondence**: **Mohammad Saleem, PhD**, Associate Professor, Molecular Therapeutics and Cancer Health Disparity Lab, Department of Urology, Masonic Cancer Center, University of Minnesota, Minneapolis, Cancer and Cardiovascular Research Building, 2231, 6th St. SE, Minneapolis, MN, 55455, Phone: 612-626-0109, msbhat@umn.edu.

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Conclusion: We suggest that *ROBO1* status forms as predictive biomarker of outcome in high-risk populations such as African-Americans and DOCK1-targeting therapy has a clinical potential for treating metastatic-CaP.

Keywords

African-American; ROBO1; prostate cancer; DOCK1; DECIPHER

Introduction

The principal cause of Prostate cancer (CaP)-associated deaths in patients is the metastasis and disease recurrence [1]. PSA is a standard clinical biomarker used for detecting biochemical recurrence in CaP patients [2, 3]. However, its utility as a marker of recurrence fails in many cases such as small-cell carcinoma, neuroendocrine-CaP where the recurrent disease is not necessarily dependent on androgen receptor (AR) the activator of PSA. Furthermore, the baseline PSA levels are different in patients when compared among different ethnicities [2, 4]. Necessitated by the lack of reliable early warning system, researchers/clinicians have resorted to biopsy-genomic tests which have recently emerged as game-changers in the field of CaP diagnosis and prediction. Recent advances in genomic-based classifications of patient populations such as the DECIPHER-test are proving beneficial to clinicians in the diagnosis and prediction of outcome [4, 5]. The decipher test is reported to predict early metastasis by employing an oligonucleotide microarray to generate an RNA expression signature after RP [4, 5]. Nevertheless, there is a need for identification of predictive markers which would broaden the applicability of genetic-testing (such as DECIPHER) for underserving sub-populations who remain undetectable by conventional diagnostic tools. Based on ethnicity, such a large high-risk sub-group are African-Americans [6]. It is important to note that even African-Americans are not a genetically homogenous population. Thus, a need exists to (i) identify high-risk sub-population among African-Americans and (ii) develop therapies that are effective in patients regardless of the race, however, perform well in African-American patients. This is because African-American CaP patients are reported to exhibit worse outcomes in response to therapies (taxane, hormone, or androgen deprivation) than Caucasians [7]. To identify such therapies, it is important to understand the mechanism underlying CaP progression. ROBO1 is a member of the roundabout immunoglobulin superfamily of proteins that actively participates in the cell motility and migration during embryogenesis and organogenesis [8, 9]. We previously showed that ROBO1 is a molecular break critical for indolent tumors to acquire metastatic phenotype particularly in African-American CaP models [9]. DOCK1 binds to the cytoplasmic domain of ROBO1 transmembrane receptor and is highly required for regulating cytoskeleton integrity of prostate epithelial cells, and the loss of ROBO1 poses a risk of activating DOCK1 as a growth promoter, and inducer of RAC1 in cells [9, 10]. The DOCK family proteins are evolutionarily conserved, Rac-specific GEFs containing the lipid-binding DHR-1 (DOCK homology region-1) and the catalytic DHR-2 domains [10]. DOCK1 functions as a Rac-specific GEF and regulates phagocytosis, myoblast fusion, and cell migration [10, 11]. By employing DECIPHER-genomic test of patients, *in vitro* and *in vivo* metastasis models (Zebra-fish), this study investigates $ROBO1^{-}/DOCK1^{+}$ signature as

predictive of poor outcome and predisposition for metastasis in high-risk patients and tests the therapeutic utility of DOCK1-inhibitor for metastatic CaP.

Materials and Methods

Cell lines, tissue specimens, antibodies and chemicals:

The description is provided in Supplementary methods file 1.

Prostate-DECIPHER genomic Classifier test:

This study adheres to the REMARK criteria for the evaluation of prognostic biomarkers. This study profiled RNA of primary CaP specimens from patients treated with radical prostatectomy (RP) at the University of Minnesota. After exclusion for tissue unavailability and quality control, the study consisted of 228 patients. To conduct DECIPER- genomic test, the total RNA was extracted, labeled and hybridized to a human Exon 1.0 St. GeneChips in a CLIA-certified facility (Decipher Biosciences, San Diego, CA). The quality control and normalization was performed using Affymetrix Power Tools and Single Channel Array Normalization (SCAN) algorithm [12]. A whole-transcriptome RNA-sequencing, (46 000 genes & non-coding RNA) was performed by the published method [13]. The DECIPHER-test data was classified and further analysis was performed at the Institute of Informatics. The CAPRA-S-score was calculated using pre-surgical PSA, pathological GS, surgical margin, extracapsular extension, seminal vesicle and lymph-node invasion. The GC scores are based on the predefined Decipher-classifier. The data was classified as risk groups (low, intermediate, high) [14].

ROBO1 promoter methylation assay:

This was performed as per the methods published by us and Dallol *et al* [15]. The detail is provided in Supplementary methods file 1.

qRT-PCR:

The primers and qRT-PCR conditions for ROBO1 were previously described [9].

DOCK1 inhibitor treatment:

ROBO1^{+ve} and ROBO1-deficient CaP cells were treated with (0–100 μM concentrations of CPYPP (DOCK-inhibitor) for 24h. The cell viability was evaluated by using an MTT assay as described [16]. For biochemical analysis, the cells were treated with sub-lethal dose (10μM) of inhibitor (based on IC_{50}). At 24h time-point, cells were processed for whole cell lysate, nuclear and cytoplasmic lysates as described [16, 17].

Transfections, luciferase reporter activity, immunostaining, immunoblot analysis, and transmigration assays.

All the experiments were performed as described previously [16, 17].

Zebrafish metastasis model:

Cancer cells were trypsinized, counted, and labeled with Cell Tracker Orange CMTMR (Invitrogen, Carlsbad, CA, USA). MDA-PCa2b cells were suspended in PBS containing DNase I and heparin and were microinjected into the pericardium of 72 hpf Tg(fli:GFP) zebrafish as previously described [16, 18]. Embryos showing cancer cells in the bloodstream after injection were selected, put in 34°C embryo water for 24 h, and then imaged on a Zeiss-ApoTome fluorescent microscope using standard FITC and dsRed filter sets.

Statistical Analysis:

Student's *t-test* for independent analysis was applied to evaluate differences between the treated and untreated cells by using PRISM statistical software. A p-value of $*p < 0.05$ were considered statistically significant.

Results

ROBO1 in normal, PIN, primary CaP and Metastatic tumors (Mets).

We previously reported the expression of ROBO1 in the primary tumors and cell lines of African-American-origin [9]. We asked whether cell-model data is relevant to clinical conditions and a difference in ROBO1 exists between primary and metastatic-CaP. ROBO1 protein has various isoforms, therefore we tested several commercial anti-ROBO1 antibodies to detect full-length (250KD) protein. The anti-ROBO1 antibody (cat#VPA00168T) performed the best for immunoblot assay (Supplementary Figure 1A). For immunohistochemical (IHC) applicability, we tested this antibody in Histogel-embedded cell models. We found strong immune-positive staining in prostate cell line RCC7T/E and scant or no staining in MDA-PCa2b (Figure 1A). These data were in agreement with our previous findings [9]. We show the immune-specificity of the anti-ROBO1 antibody in human tissues (Jejunum and kidney) (Figure 1A). Next, we performed the IHC of normal adjacent to tumors (NAT), prostate intraepithelial neoplasia (PIN), primary-CaP (Gleason Grade Group I-IV) and metastatic tumors (Mets) from African-American and Caucasian patients. African-American cases (n=88) included NAT (n=30), PIN (n=16) and; primary-CaP (n=32; Grade Group I/II (n=16) and III/IV (n=16)) and Mets (n=10). The Caucasian cohort included NAT (n=23), PIN (n=13) and primary-CaP (n=23) comprised of Grade-Group I/II (n=17) and III/IV (n=6) and Mets (n=17). In agreement with our previous report, we found the loss of ROBO1 protein in primary-CaP when compared to normal (Figure 1B). The loss of ROBO1 was noted prominent in African-Americans in an order NAT>PIN>Grade I/II > Grade IV >Mets (Figure 1Bii). We scored immunostaining on a scale of 0–4 on the basis of clinically acceptable histopathology protocol viz., 0=none, 1=weak, 2=moderate, 3=strong, 4= very strong. The immunostaining distribution score was as follows:

Immunostaining distribution (normal prostate tissues): The average immunostaining score for ROBO1 in NAT for African-Americans was recorded as 1.8 ± 0.09 (mean ± SEM) compared to 1.8 ± 0.1 in Caucasians (Figure 1Bii–iii). The staining score for ROBO1 in NAT specimens was strong in 13% in African-American cases (n=30) compared to 35% of Caucasian counterparts (n=23) (Table 1).

Immunostaining distribution (PIN tissues): If all cases put together for each race, no significant difference in the average score is observed between African-Americans and Caucasian PIN. However, the intra-group distribution shows a stark difference between African-American and Caucasian cases. The ROBO1 immunostaining in PIN tissues (n=16) tested strong only in 6% and weak in 63% in African-Americans (Table 1). However, Caucasian PIN (n=13) exhibited ROBO1-staining strong in 31% and weak in 23% cases (Table 1).

Immunostaining distribution (cancerous prostate tissues): African-American primary CaP (n=32) displayed “No staining” in 53% and weak in 44% cases (Table 1). On the Contrary, 100% of Caucasian cases tested ROBO1-positive. The staining score was moderate in 16% and weak in 74% cases (Table 1). Notably, African-American cases exhibited almost complete loss of ROBO1 in Grade-III/IV CaP whereas no such difference was observed in counterpart Caucasians (Figure 1Bii, Table 1). When compared with NAT, the average ROBO1-staining score in African-Americans was significantly lower in both Grade-Group I/II (0.6 ± 0.125) and Grade-Group III/IV (0.125 ±0.05) representing a 2.8 and a 14.4-fold decrease, respectively *(**p<0.001*, Figure 1Bi). For Caucasians, the Grade-Groups I/II and III/IV tumors recorded an average score of 1.0 ± 0.10 and 0.75 ±0.17. Comparing of NAT with Grade III/IV, the African-American cases exhibited 14.4-fold decrease whereas Caucasians exhibited 2.3-fold decrease (***p<0.001*) (Figure 1Bii–iii).

Immunostaining distribution (Metastatic tumors or Mets): The IHC of Mets showed positive staining of ROBO1 in 100% Caucasian cases whereas 90% of Mets of African-Americans (n=10), exhibited negative staining for ROBO1 (Figure 1Ci). Notably, 10% remaining Mets showed very scant immunostaining. These data suggest that the ROBO1 is lost in African Americans more than Caucasians during CaP progression (Figure 1Cii).

DECIPHER genomic test: Loss of *ROBO1* predicts poor outcome in patients

The prostate-DECIPHER- genomic test provides multiple algorithms predicting clinical outcomes based on RNA-Sequencing data of patient tumors/biopsies [19]. Basic Decipher-test selects the RNA expression of 22 gene markers to predict outcome and patients are classified as ‘low’,’ average’, and high risk [19]. The DECIPHER test is now made highly advanced by adding new algorithms such as Genomic Gleason algorithm, and CAPRAS algorithm, etc. We investigated the expression of *ROBO1* in relation to algorithmic outcomes predicting clinical status by utilizing DECIPHER-test of 228 patients who underwent RP-treatment at our institute.

(i) Genomic Gleason: Decipher has developed an algorithm (Genomic Gleason) that identifies Gleason Score and uses the genomic data as input and classifies samples as “High Grade” (4+Grade) and “Low Grade” (<4). To validate if ROBO1 performs-well in discriminating cancer grades, we analyzed ROBO1 status in Minnesota-Cohort patients classified by Decipher-Genomic-Gleason algorithm. We observed low-ROBO1-expression in highly-aggressive classified tumors and a high ROBO1 indicating low-grade CaP (Figure

2A). These data indicate validated ROBO1 performing as an independent phenotype-distinction marker.

(ii) Extra-prostatic extension (EPE): The EPE is a prostate tumor stage where the tumor surpasses the prostate borders and is the beginning of the metastasis [20]. Decipher-algorithm successfully predicts the risk of EPE in patients. We tested the Decipher-classified EPE cases for *ROBO1* expression and found that CaP patients classified as 'No EPE' demonstrated higher levels of *ROBO1* than patients predicted as high-risk EPE (*p=0.038*). These data support the hypothesis that loss of *ROBO1* is critical for localized tumors of becoming invasive.

(iii) Risk of distant metastasis: Decipher CaP-test developed at Mayo Clinic selects gene markers (22 coding and non-coding genes) to predict the risk of metastasis after initial treatment. The analysis of DECIPHER metastasis-risk classified tumors showed a significant (*p=0.0001*) association of lower risk of metastatic prevalence to higher *ROBO1* expression in CaP-patients. Conversely, a low *ROBO1* expression predicted a higher risk-of metastasis (Figure 1C).

(iv) Risk of recurrence: CAPRAS is a risk assessment methodology and Decipher-algorithm predicts CAPRAS Score using genomic data as input [21]. This algorithm has a binary output of "Higher Grade" and "Lower Grade" S-Score. We evaluated CAPRAS-score in our clinical cohort. We found a high expression of *ROBO1* in CaP-patients with lower CAPRAS S-Score (p=4.54E-5). Conversely, patients with high-CAPRAS score exhibited low-*ROBO1* (Figure 1D).

(v) Risk of CaP-associated mortality: Penney *et al* identified an mRNA signature to improve prediction of lethal CaP among men with Gleason 7 tumors, the most common CaP- grade, and the most indeterminate in terms of prognosis [22]. DECIPHER-testing used this signature to identify our clinical cohort as average, low and high mortality risk. We observed that the *ROBO1* is significantly (*p=0.038*) low in patients predicted with high-risk of CaP-specific mortality (Figure 2E). Conversely, patients predicted with a lower-risk of mortality harbored high *ROBO1* expression in primary tumors (Figure 2E). Overall, DECIPHER data aligned with the biological narrative indicate that *ROBO1*-negative feature of tumors is associated with high-risk to develop aggressive tumor type with poor outcome in CaP patients.

Low expression of *ROBO1* is associated with poor survival

We asked if the findings in Minnesota cohort are reproducible in other clinical cohorts. We performed data mining using an ULCAN platform and analyzed the PRAD-TCGA clinical data. The data obtained from 503 patients with prostate primary tumor (Caucasian n=497 and African-American n=6) compared with 52 normal tissues showed reduced expression of *ROBO1* in primary CaP (Figure 2F). We found that CaP-patients with low/medium ROBO1 expression combined with high-Gleason present poor survival in CaP patients (Figure 2H).

ROBO1 promoter is methylated in CaP

The epigenetic changes in tumor genome including modifications in the chemical structure of DNA such as methylation are reported as a contributing factors in tumor suppression and CaP progression [23]. Several reports have identified individual differentially methylated CpG sites and methylation signatures associated with CaP-progression [24]. Previously we reported methylation of *ROBO1* gene promoter in African-American cell model MDA-PCa2b whereas no *ROBO1*-methylation was observed in Caucasians cell models [9]. We asked if DNA methylation causes cancer progression in the ROBO1-deficient cases, particularly African-Americans. To explore an association between methylation and loss of *ROBO1* expression, we analyzed NAT and prostate tumors with known *ROBO1* expression (Figure 3A). Next, we evaluated the methylation status of the *ROBO1* promoter in prostatic tumors as described [9]. We amplified the DF3 region of the promoter of *ROBO1* (327bp), as a hotspot for methylation in patients (Figure 3C). We detected fragmented DNA's (due to methylation at 340 and 264 bp) (Figure 3D). A higher percentage of African-American cases exhibited methylation in the *ROBO1* than Caucasians (Figure 3D). Notably, 70% of NAT exhibited methylation of *ROBO1* in African-Americans compared to 10% of such cases in Caucasians (Figure 1E). The methylation of ROBO1 in normal regions suggests the predisposition of African-Americans for CaP metastasis.

Furthermore, we observed that methylation of *ROBO1* is highly prevalent in African-American CaP-patients (80–90%) than Caucasians (Figure 3Ei). Notably, 100% African-American cases regardless of Gleason Score (3+4 or 5+5) exhibited *ROBO1*-methylation whereas Caucasian patients exhibited ROBO-methylation in 60–80% high Gleason-grade tumors (Figure 3Eiii). These data suggest that *ROBO1*-methylation might be occurring late in Caucasians during progression whereas this could be an early event in African-Americans. Finally, we analyzed methylation data of PRAD-TCGA patient cohort (n=552) and found a higher degree of *ROBO1* methylation in primary CaP than normal tissues (Figure 3F).

DOCK1/Rac1 signaling in ROBO1-deficient prostate tumors

We posit that ROBO1-DOCK1 interaction is a natural controlling mechanism of Rac1 activity in normal cells. The model of our hypothesis is presented in Figure 4A. However, due to the loss of ROBO1, the DOCK1 in free from is hyper-active, promotes GDP-GTP nucleotide exchange and activates Rac1 which in turn triggers the β-catenin signaling [25]. It is known that activation of Rac1 induces loss of adhesion to the extracellular matrix and increased invasiveness through controlling actin cytoskeleton organization. We previously showed that ROBO1 regulates Rac1 activity in prostate epithelial cells [9]. The role of DOCK1 as a critical checkpoint for Rac1 in prostate tumorigenesis has not been studied yet. This report is the first attempt to evaluate the significance of DOCK1 in human CaP.

Prostate-DECIPHER genetic test for DOCK1: Decipher has developed algorithms that identify multiple cancer sub-types results. These algorithms are developed to distinguish between primary and metastatic cancers. Using our Minnesota Cohort (n=228 patients), we determined the status of *DOCK1* in primary CaP cases using the DECIPHER-classifier test analysis. We found that DOCK1 expression is significantly (*p=0.039*) higher in African-

American (n=9) CaP patients than Caucasian counterparts (n=146) (Figure 4Bi). However, the number of African-American patients is less and warrants further investigation in a large cohort of this population.

IHC analysis in primary and metastatic tumors for DOCK1: We selected ROBO1-deficient high-grade primary prostate tumors and Mets for determining the level of DOCK1 protein by employing the IHC method. We found that primary tumors and metastatic tumors exhibit a high level of DOCK1 protein (Figure 4Bii). The IHC data are consistent with the DECIPHER-genetic test of the Minnesota patient cohort. When compared, Mets exhibited a higher level of proteins than primary tumors (Figure 4Bii & Supplementary Fig. Bii). We also found that DOCK1 levels in NAT are almost equal to low-stage primary CaP and confined to the cell membrane (Supplementary Fig.1Bi). However, it is notable that high-grade prostate carcinoma and Mets exhibited a high degree of DOCK1 protein in the cytosol as well as in nuclei of cells suggesting disintegration of ROBO1/DOCK1 complex in high-stage or metastatic disease. This was confirmed by the testing distribution of DOCK1 in ROBO1-negative metastatic cell lines MDA-PCa2b and VCaP by using immunoblotting and confocal microscopy (Figure 4C & Supplementary Figure 1C).

Rac1 predicts the risk of distant metastasis: Based on the DECIPHER-genetic test of primary prostate tumors (RP-treated) which classified the Minnesota Cohort (n=228) into three groups (i) average-risk, low-risk, and high-risk, we measured the risk of metastasis with respect to *Rac1* expression. We observed a significantly ($p=0.006$) higher risk of metastatic prevalence in CaP-patients with high *Rac1* expression (Figure 1Bii). Conversely, cases with low *Rac1* expression were predicted to have a low-risk of distant metastasis ($p=0.001$) (Figure 1Bii).

IHC analysis in primary and metastatic tumors for Rac1: We determined the levels of Rac1-GTP (the active form Rac1), in African American and Caucasian tissue specimens. First, in order to validate the specificity of the anti-Rac1-GTP antibody we performed the IHC analysis of ROBO1-sufficient primary cell (RC77T/E) and ROBO1-deficient metastatic cell model MDA-PCa2b embedded in histogels. Whereas RC77T/E exhibited positive weak-moderate staining, the MDA-PCa2b exhibited very strong immunostaining for Rac1-GTP (Supplementary Figure1D). This data indicated an increased level of Rac1 activity in metastatic stages of CaP. Second, we performed the IHC analysis for Rac1-GTP in African-American and Caucasian specimens, which included NAT, primary tumor grades I/II and III/IV, and Mets. The results showed that the levels of Rac1-GTP were increased in primary tumors compared to NAT specimens in both African-American and Caucasian (Figure 4Dii). However, when we compared tumor grade I/II with III/IV, we observed a progressive increase in the Rac1-GTP levels in African-American cases whereas Caucasian specimens exhibited no such difference (Figure 4Dii–iii).

Based on the scoring patterns, a significant difference in Rac1-GTP protein expression was observed between normal prostate and CaP specimens (Table 2). In African American patients the immunostaining pattern in NAT specimens (n=27) for Rac1-GTP was noted as 56% weak, 37% moderate, and 7% strong (Table 2). In Caucasians, the Rac1-GTP immunostaining pattern in NAT specimens (n=22) was observed as weak in 41% and

moderate in 59% (Table 2). In Caucasians, the Rac1-GTP specific immunostaining in PIN (n=12) specimens was noted weak in 83% and moderate in 17% cases (Table 2). In African-Americans, the Rac1-GTP positive immunostaining pattern in PIN specimens (n=16) was weak in 63% and moderate in 37% (Table 2). In African-Americans, the staining for Rac1-GTP in primary CaP specimens (n= 33) showed weak staining in 9%, moderate in 49% and strong in 42% (Table 2). However, Caucasian primary tumors (n=20) recorded a staining pattern as weak in 5%, moderate in 85% and strong in 10% (Table 2). Moreover, in African-American specimens the increment of Rac1-GTP was markedly higher in tumor grade III/IV compared to grades I/II. As anticipated, the loss of ROBO1 was inversely associated with Rac1-GTP expression. These data suggest that Rac1-GTP expression is steadily increased during progressive CaP-stages in African-Americans whereas Caucasian counterpart patients do not exhibit a strong correlation between Rac1-GTP and disease stage.

Rac1-induced cytoskeleton disturbance causes the loss of E-cadherin and induction of β-catenin in ROBO1-deficient cells [9]. The IHC analysis shows that E-cadherin levels are deceased whereas β-catenin levels (nuclear) are increased in ROBO1$^{-\text{deficient}}$ Mets in African-Americans (Figure 1E). African-American cases exhibited low-ROBO1 negatively correlated to the nuclear β-catenin levels (Figure 1D–C). These data support the notion that the ROBO1-deficiency causes activation of Rac1/β-catenin (which promotes metastasis), particularly in African-Americans.

Pharmacological inhibition of DOCK1 for treating metastatic CaP

Metastatic CaP is mostly recognized by its resistance to conventional treatments such as androgen-deprivation therapy (ADT) and chemotherapies. The DECIPHER-genetic testing of RP-treated patients showed a higher of risk of metastasis in ROBO1$^{-\text{deficient}}$ CaP patients (Figure 2). We determined if *ROBO1* deficiency predicts the outcome of ADT in CaP patients based on DECIPHER-classification of Minnesota cohort (n=228 patients). Patients were classified into two groups (i) highly-responsive and (ii) average-responsiveness. We observed that the probability of high-responsiveness is significantly decreased in ROBO1-deficient patients ($p=0.06$; Figure 5A). This is consistent with our data where the same cohort exhibited an increased risk of metastasis in *ROBO1*-deficient patients. The data presented in figures 1–5A strongly supports the hypothesis that *ROBO1* loss has implications in CaP disease which is manifested in the form of hyperactivation of DOCK1-mediated Rac1 activity that in turn promotes the metastatic spread of malignant cells to distant organs. Therefore, DOCK1 presents an ideal target for therapies to treat CaP metastasis, particularly ROBO1-deficient subtype. DOCK1 mediates nucleotide exchange activity of Rac1 and stabilizes Rac1-GTP protein levels in cells [25]. Recently, a small molecule (CPYPP,) has been discovered that inhibits DOCK1 binding to its substrate such as Rac1 [26]. We performed following assays with CPYPP using *in vitro* and *in vivo* models:

(i). Lethal and effective-dose identification: To measure a safer and effective dose, we performed an MTT-based cell viability assay where *ROBO1*-$^{\text{deficient}}$ metastatic cells (MDA-PCa2b/VCaP) and *ROBO1*$^{+ve}$-basal level expressing primary cell (RCC7T/E) were treated with CPYPP (1–100μM) for 24h. The MTT analysis showed that DOCK1 inhibitor treatment caused a reduction in cell viability in a dose-dependent manner (Figure 5B). The

effective concentration (EC_{50}) at which 50% cell growth was inhibited for *ROBO1*-deficient cells was noted as 31–35μM whereas for *ROBO1*-positive cells it ranged from 16μM (Figure 5B).

(ii). Trans-endothelial membrane tumor cell migration: We tested the potential of metastatic cells in crossing the vascular barrier using an *in vitro* model of extravasation. This model comprises a transwell culture plate containing a polycarbonate membrane covered with a layer of human endothelial cells. Under drug-free conditions, tumor cells with invasive potential migrate across the endothelial cell lining and polycarbonate membrane. The cells that crossover to the other side of the membrane are detected by crystal violet staining and counted under the microscope. Based on the cell-viability assay, we selected a sub-lethal dose (10μM) to test its efficacy against the invasive potential of live metastatic *ROBO1*-deficient MDA-PCa2b cells. The vehicle (DMSO) treated cells did display a 100% capability of migrating through endothelial lining after growing in transwell for 24 h. However, treatment of CPYPP caused a reduction, of 45% in the number of transmigratory MDA-PCa2b cells (Figure 5C).

(iii). Effect of DOCK-inhibition on metastasis of cells in vivo: Because the inhibition of DOCK1 resulted in decreased transmigration of metastatic cells under *in vitro* conditions, we asked if this data could be translated *in vivo*. During metastasis, tumor cells intravasate into the bloodstream from their primary site, migrate through the vascular system, and invade distant tissues by extravasating from the bloodstream. Due to the translucence of zebrafish embryos and the histological and genetic commonalities shared by zebrafish and human vasculature, this animal represents an appealing vertebrate model to study metastasis [27]. To test the efficacy of DOCK1-inhibitor (CPYPP) in inhibiting the metastasis of MDA-PCa2b CaP cells, we used Tg(fli:GFP) transgenic zebrafish (which express GFP under the control of a vasculature-specific promoter) [16, 18]. Either control or CPYPP-treated (10 μM for 24 h) MDA-PCa2b cells were transiently labeled with a fluorescent tracker dye and microinjected into the bloodstream of 72 hours post-fertilization (hpf) Tg(fli:GFP) zebrafish embryos. The following day at 24 h post-injection of cells, the embryos were imaged using a fluorescence microscope. In the control group, we observed MDA-PCa2b cells in the extravascular space, whereas CPYPP pre-treated MDA-PCa2b cells remained in the vasculature (Figure 5D). These data demonstrate that pharmacological inhibition of DOCK1 reduces CaP cell migration/metastasis in a vertebrate model.

(iv). Effect of DOCK1 inhibition on downstream signaling: The important downstream target of DOCK1/Rac1 signaling is β-catenin in CaP cells [9]. We show that *ROBO1*-deficient prostate tumors and DOCK1-activated cells harbor elevated nuclear and free β-catenin (Figure 4). We evaluated the effect of the CPYPP-treatment on the TCF-transcriptional activity (indicator of nuclear β-catenin activity) by employing a TOP-FLASH-reporter assay in RCC7T/E and MDA-PCa2b cells. Treatment with CPYPP (10μM) decreased the reporter-activity in CaP-cells however a significant inhibition in metastatic MDA-PCa2b cell line was observed (Figure 5Ei–ii).

Next, we validated these data by confocal microscopy and evaluated the expression of β-catenin and Rac1-GTP. We found that the CPYPP-therapy causes a marked decrease in the

levels of nuclear β-catenin and Rac1-GTP in the primary-cell model RCC7T/E, however the effect was more marked in metastatic cells in ROBO1-deficient cells (MDA-PCa2b, VCaP) (Figure 5F). Whereas CPYPP caused a decrease in Rac1-GTP, the effect on β-catenin was marginal in PC3 cells suggesting preference of CPYPP therapy for *ROBO1*-deficient tumor cells (Supplementary Figure 1E). The VCaP is the only Caucasian cell line that is *ROBO1*-negative and showed similarity to the African-American metastatic model MDA-PCa2b. Because, we observed a straight effect of CPYPP on each stage of DOCK1/Rac1-GTP/β-catenin pathway in ROBO1-deficient cells, we suggest that DOCK1-targeting could be an ideal strategy to treat *ROBO1*-deficient metastatic CaP, particularly in African-American men. To summarize, ROBO1-loss in biopsies was found to be a critical predictor of recurrent, metastatic and drug-resistant CaP. Furthermore, DOCK1/Rac1 pathway was identified as a target for treating metastatic CaP, including high-risk patients such as African-Americans who commonly display *ROBO1*-deficiency.

DISCUSSION

As per the American Cancer Society annual report, 29, 570 new cases of CaP and 5,350 cases of CaP-related deaths were reported in African-American men in the United States in the year 2019 [28]. The CaP incidence rate is 76% higher and the mortality rate is 2.3 times higher in African-American men than Caucasians [29]. It becomes imperative to understand the molecular events or alterations which contribute to the disparity in the disease-outcome between two ethnic groups. These would be important in developing specific therapies and biomarkers which would benefit all patients including African-Americans. This study identified the significance of *ROBO1*-deficiency/DOCK1^{+ve} signature as a biopsy-biomarker that would successfully predict the risk-of recurrence/therapy failure and DOCK1-inhibitor as potential therapy for aggressive CaP that would preferentially benefit African-Americans patients.

ROBO1 protein is involved in cell motility and migration during organogenesis. Malfunctioning of ROBO1 due to missense mutations has been associated with neurological disorders in humans [30]. Since our report in 2014, several studies have emerged focused on the role of ROBO1 in human cancers [9, 31]. A few studies suggested ROBO1 as a cancer gene, however majority of published reports firmly support the tumor suppressor role of *ROBO1*. We reason that the presence of ROBO1 protein in primary tumors (including prostate tumor) is acknowledged, however, its loss would indicate a progression towards metastasis. Aided by the DECIPHER-genetic classifier test, the current study provides strong evidence supporting ROBO1 as a predictive marker of metastasis and recurrence in CaP-patients who undergo primary treatment. Although not very common, *ROBO1* loss could also be an indicator of aggressive CaP in Caucasian patients. Nevertheless, we believe that African-American patients form a large sub-group that is prone to *ROBO1* loss and is at risk of recurrence (after RP). Given the fact that there is no reliable biomarker of disease recurrence specifically for African-Americans, ROBO1-biopsy testing would be highly beneficial to this high-risk patient population.

Although genetic data, clinical data, and cell-based data provide a strong basis for supporting the loss of *ROBO1* as a predictor of CaP aggressiveness, its relevance would not

be complete without knowing the reasons underlying the loss of expression. Wei et al reported that *ROBO1* expression is repressed by Sp8 and Sp9 transcription factors in normal brain tissues [32]. A recent study by Gorla *et al* showed a post-translational modification of ROBO1 protein by Ndfip1/2 protein-mediated ubiquitination in neural tissues/axons [33]. Epigenetic changes such as methylation are frequently involved in repression of expression and tumor progression. Several tumor suppressor genes such as *ZNF545*, *Jagged1*, *Znf382* and *LATS2* have been reported to harbor hypermethylation which in turn causes their loss of expression [34]. Dallol *et al* reported that the promoter region of *ROBO1* has the propensity of hypermethylation in human cells [15]. We reported *ROBO1* gene methylation exclusively in African-American metastatic cell line MDA-PCa2b [9]. The significance of current study is that it validated methylation as a possible reason for the loss of *ROBO1* in CaP patients who displayed reduced mRNA expression of *ROBO1*. Our findings were further corroborated by the data of a large-patient cohort (TCGA-PRAD clinical study). These data open a window of opportunity for testing specific demethylation agents for restoring the expression of ROBO1, however, mixed results of demethylation agents in clinics warrants an alternate strategy to contain the fallout of the loss of ROBO1 in tumor cells.

We had reported that loss of *ROBO1* causes disintegration of the DOCK1 complex that in turn triggers loss of E-Cadherin and activation of Rac1 signaling in CaP cell models [9]. Our study is corroborated by a report by Xia *et al* showing loss of ROBO1 as a critical event underlying EMT of neoplastic cells [35]. Tan *et al* reported that the ROBO1-C3 cytoplasmic domain has affinity to regulate E-cadherin-dependent EMT in cells [36]. Based on these data, we suggest that DOCK1 forms an excellent target for ROBO1-deficient metastatic CaP. The efficacy of the DOCK1 inhibitor tested in a zebra-fish model (ideal to monitor drug efficacy in real-time) and in the transmigration model (ideal to monitor in real-time the cancer cell migration) lends clinical relevance to our findings. To conclude, the ROBO1-biopsy marker would improve the performance of the DECIPHER-test in predicting the disease outcome in African-American patients. ROBO1-biopsy testing may help in reducing the overtreatment and unnecessary morbidity in populations such as African-Americans. The outcome of DOCK1-targeted therapy suggested a novel agent for the treatment of metastatic CaP that has the potential to benefit a larger section of patients including those *ROBO1*-deficient patients (such as African-Americans) who are at high-risk to develop aggressive disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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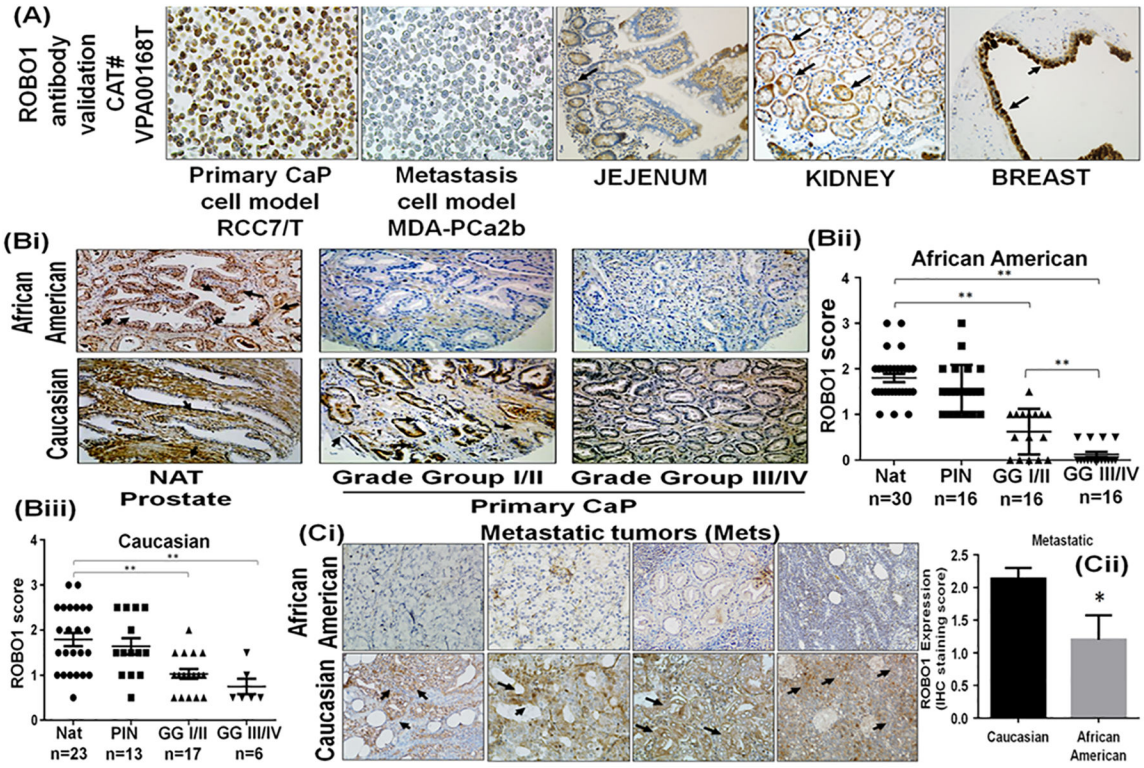

Figure 1: Comprehensive immunohistochemical analysis of ROBO1 in primary and metastatic tumors of African-American and Caucasian patients.

(A) Shows the specificity of the anti-ROBO1 antibody in detecting full-length ROBO1 protein in *ROBO1*$^{+ve}$ RCC7T/E cells, *ROBO1*$^{-deficient}$ MDA-PCa2b cells, in human tissues by employing IHC assay. **(Bi & Ci)** shows the expression of ROBO1 in African-American primary tumors and Mets. The arrow-pointed brown color staining indicates ROBO1-positive regions in NAT, primary tumors (Gleason Grades I, II, III, and IV) and Mets. **(Bii-iii)** Scatter graphs show the individual IHC-immunostaining score (on a scale of 0–4) in tumor specimens. (**Cii**) The histogram shows the comparison in ROBO1 immunostaining score between the African-American and Caucasians.

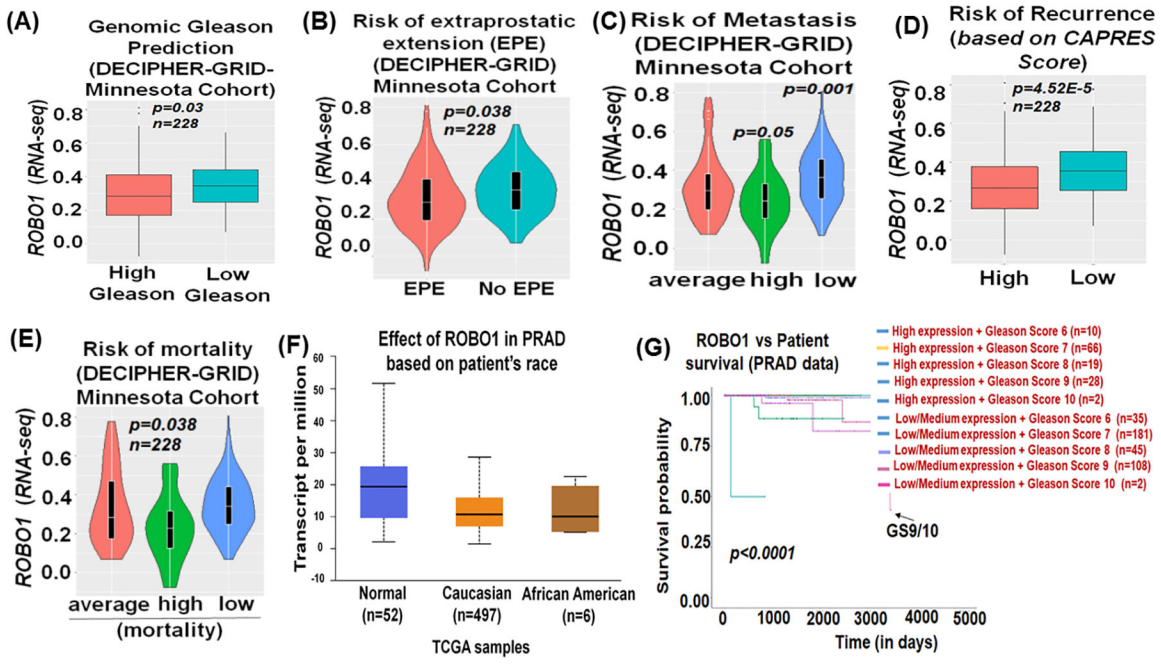

Figure 2: *ROBO1* as predictor of risk of aggressiveness, recurrence, metastasis and mortality aided by Prostate-DECIPHER-genome test.

(A) Genomic Gleason is an algorithm based on a set of genes predicts Gleason grade. The Box plot shows the association of *ROBO1-loss* as a predictor of high genomic Gleason in 228 RP-treated CaP patients (**B-C**) The violin graphs show the association of *ROBO1-loss* as a predictor of (**B**) EPE and **(C)** high-metastasis in RP-treated CaP patients. (**D**) The violin graphs show the association of *ROBO1-loss* as a predictor of recurrence in RP-treated patients. (**E**) The violin graphs show the association of *ROBO1-loss* as a predictor of mortality in RP-treated patients. (**F-G**) Figures show the results generated from the analysis of total genome-data of prostate tumors accrued under PRAD clinical study by using the UALCAN platform (**F)** Box-plots show the quantification of ROBO1-transcription (transcript /million) in CaP patients. **(G)** The Kaplan Meier curve shows the association of *ROBO1-loss* to the survival of CaP patients with different Gleason Scores.

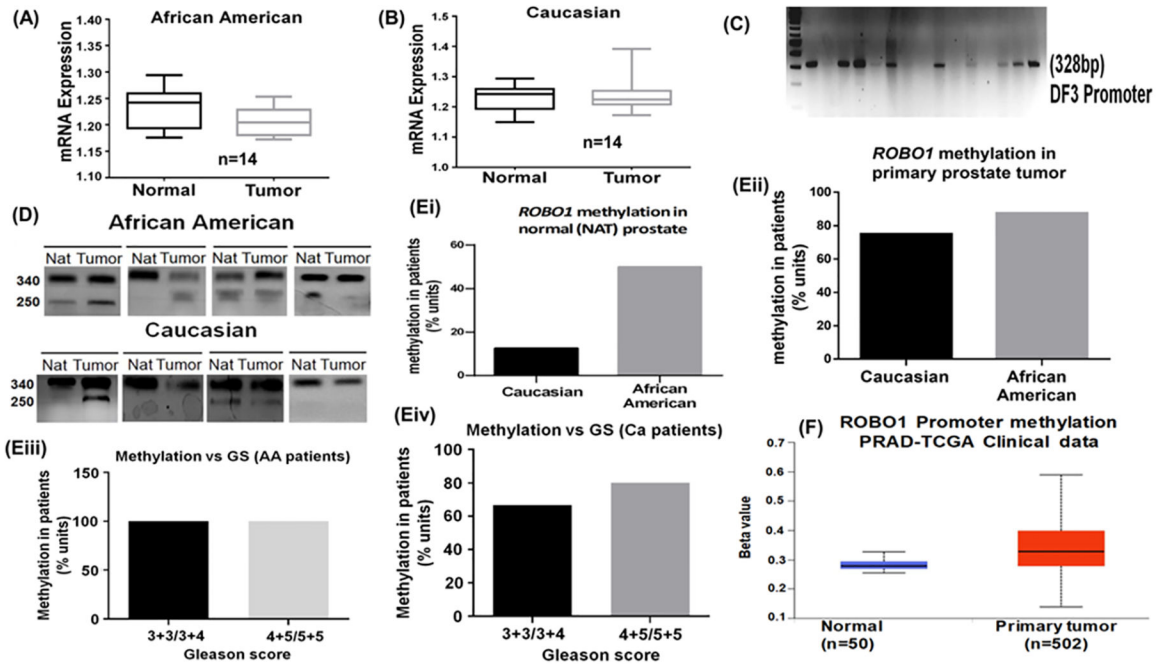

Figure 3: *ROBO1* methylation status in African-American and Caucasian prostate tumors and quantification of methylated-*ROBO1* in samples from PRAD-TCGA clinical study.

(**A-B)** Box-plots show the quantification of ROBO1 mRNA-expression in African-American and Caucasian CaP patients. **(C)** The Gel-image shows the PCR-product for the *DF3* promoter region of the *ROBO1* in human prostate samples. **(D)** The Gel-image shows the bands indicating the methylation at the *DF3*-region of the *ROBO1* gene (**E**) Histograms show the quantified value of methylation of the *ROBO1* in CaP samples. (**Ei-Eii)** Shows a comparison of the methylation of ROBO1 in NAT and primary tumors. (**Eiii-Eiv)** Shows methylation level of *ROBO1* versus Gleason Scores in primary tumors. **(F)** Histogram compares methylation of *ROBO1* in normal and CaP in patients under PRAD-TCGA study. The quantification of methylation is presented as β-value.

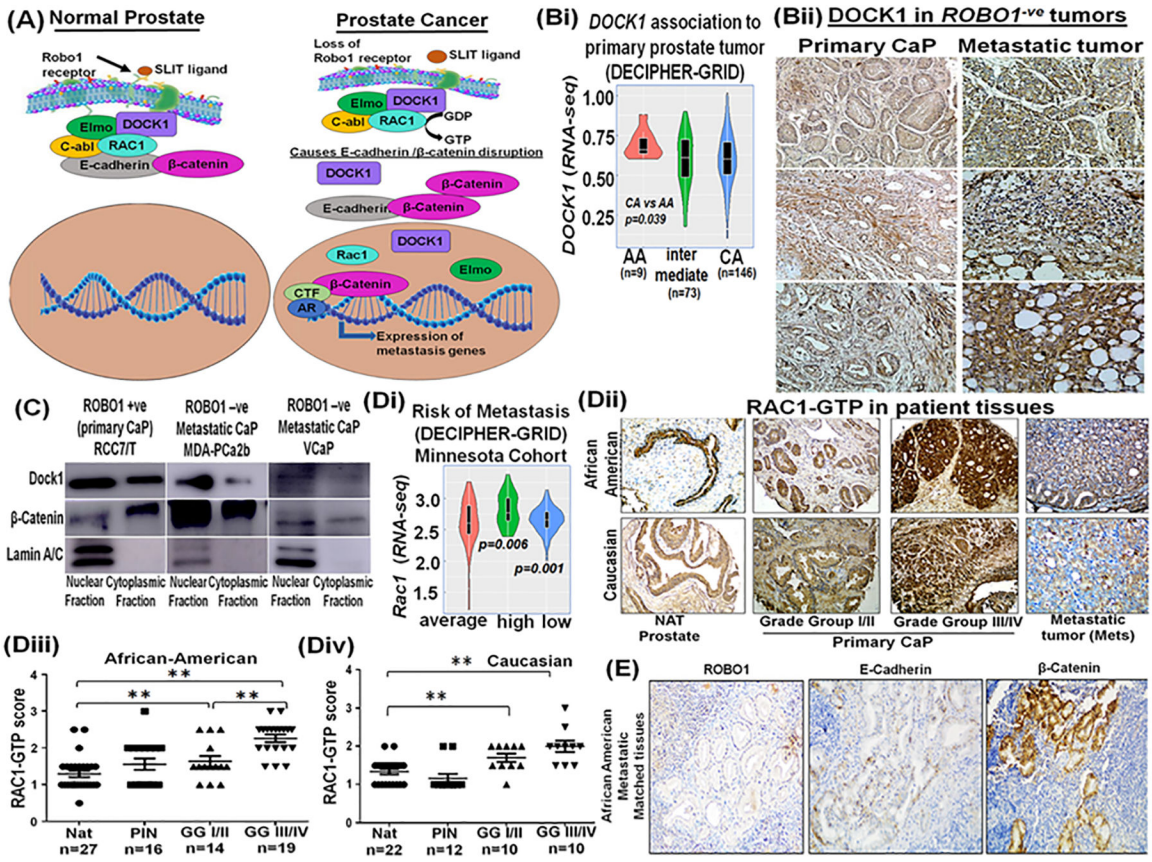

Figure 4: Clinical significance of DOCK1-regulated Rac1 pathway in disease progression and risk-of metastasis in CaP disease in African-Americans and Caucasians.

(A) The cartoon shows the model of the ROBO1-DOCK1-Rac1 molecular pathway in normal and cancerous prostate cells. (**Bi**) DECIPHER-test developed an algorithm (based on race-based molecular signatures). The violin graphs show the expression of *DOCK1* in primary tumors of RP-treated African-American and Caucasian patients using the DECIPHER-Algorithm. **(Bii)** Shows the expression of DOCK1 in African-American primary CaP and Mets using the IHC technique. The arrow-pointed brown color staining indicates DOCK1-positive regions in tissues. (**C**) immunoblot image shows the level of DOCK1 and downstream target β-catenin in the cytoplasmic and nuclear fractions of *ROBO1*$^{+ve}$ RCC7T/E and *ROBO1*$^{-ve}$ MDA-PCa2b and VCaP cell models. (**Di**) DECIPHER-test developed algorithms that predicts risk of metastasis categorized as low, average and high. The violin graphs show the association of *Rac1* expression as a predictor of metastasis in *ROBO1/DOCK1* matched patient cohort (**Dii**) shows the level of activated Rac1 protein (Rac1-GTP) in African-American and Caucasian NAT, primary tumors and Mets as evaluated by the IHC technique. The brown color staining indicates Rac1-GTP positive regions in primary prostate tumors (Gleason Scores I-IV) and Mets. (**Div-v**) Scatter graphs show the IHC-immunostaining score for Rac1-GTP (on a scale of 0–4) in tumor specimens pointing to brown staining) in African- American and Caucasians CaP tissues. **(E)** Shows the expression of ROBO1, and targets of DOCK1/Rac1 network (E-cadherin, β-

catenin) in matched tissues of African-American Mets using IHC technique. The arrow pointes to immuno-positive regions.

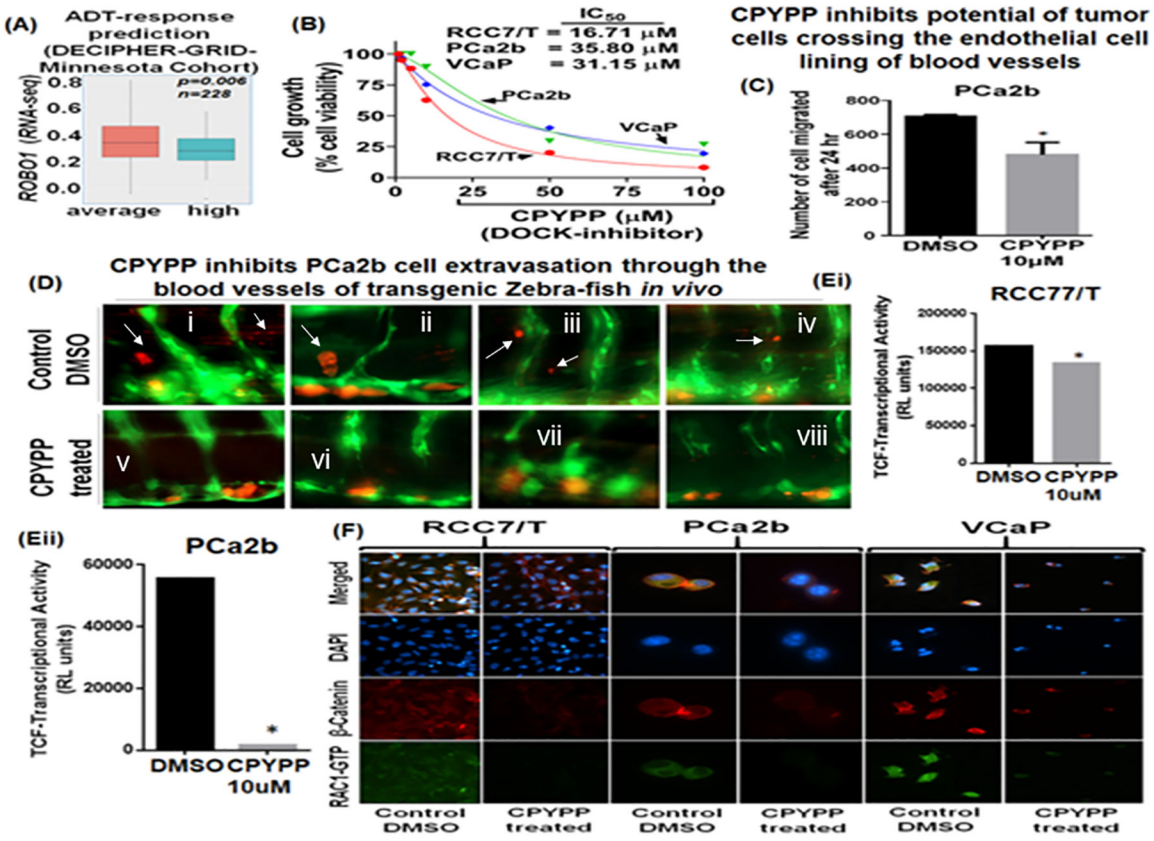

Figure 5. Testing the impact of pharmacological Inhibition of DOCK1 in ROBO1^{+ve} and ROBO1^{-ve} CaP models.

(A) (**B**) **The l**ine graph shows the growth (% viability) of *ROBO1*$^{+ve}$ -indolent (RCC7T/E,), *ROBO1*$^{-ve}$ metastatic (MDA-PCa2b; VCaP) CaP cells after treatment (0.5–100 μM for 24h) with CPYPP, an inhibitor of DOCK1, as assessed by MTT assay. (**C**) The histogram shows the efficacy of CPYPP therapy in inhibiting the metastatic potential of MDA-PCa2b cells as assessed by Endothelial-cell based transmigration assay. **(D)** Pharmacological DOCK1 inhibition with CPYPP reduces extravasation of MDA-PCa2b cells in zebrafish**. Di-iv:** Representative images of control MDA-PCa2b cells (red) that have extravasated from the vascular system (green) are shown in extravascular space. **Dv-viii**: Representative images of CPYPP-treated MDA-PCa2b cells (red) are visible within the vascular system (green) of zebrafish with no detectable extravasation events. **Di-Dviii:** n>10 zebrafish per group. Images were captured on a fluorescent microscope 10X, 0.45 NA objective, digitally magnified 2X. (**Ei-ii**) Bar-graphs show the effect of CPYPP-therapy on *ROBO1*$^{-ve}$ MDA-PCa2b and *ROBO1*$^{+ve}$ RC77/T cells as assessed by the TOP-FLASH-reporter assay (indicator of β-catenin activity) (**F**) Microphotographs show the effect of CPYPP treatment on Rac-activation (Rac1-GTP level) and nuclear β-catenin in CaP models as assessed by confocal microscopy.

Table 1.

Expression of ROBO1 in normal human prostate, PIN and adenocarcinoma specimens in African-American and Caucasians

ROBO1 staining intensity													
African-American							Caucasian						
	Total	None	Weak	Moderate	Strong	p		Total	None	Weak	Moderate	Strong	p
Total	78	17	27	29	5		**Total**	59	0	26	21	12	
NAT	30	0	3	23	4		**NAT**	23	0	6	9	8	
PIN	16	0	10	5	1		**PIN**	13	0	3	6	4	
Carcinoma	32	17	14	1	0	0.0001*	**Carcinoma**	23	0	17	6	0	0.0001*
Grade 1–2	16	5	10	1	0	0.0001*	**Grade 1–2**	17	0	12	5	0	0.0007*
Grade 3–4	16	12	4	0	0	0.0001*	**Grade 3–4**	6	0	5	1	0	0.0019*

Note: The expression of ROBO1 was evaluated as staining of the tissue as none (0), weak (0.5–1), moderate (1.5–2) and strong (2.5–3). Fisher exact test was used to examine the association between staining intensity and tissue type or staining intensity and tumor grade (for tumor specimen only).

* $p < 0.05$ was considered significant.

Table 2.

Expression of RAC1-GTP in normal human prostate, PIN and adenocarcinoma specimens in African-American and Caucasians

RAC1-GTP staining intensity												
African-American							Caucasian					
	Total	None	Weak	Moderate	Strong	p		Total	None	Weak	Moderate	Strong	p
Total	76	0	28	32	16		**Total**	54	0	20	32	2	
NAT	27	0	15	10	2		**NAT**	22	0	9	13	0	
PIN	16	0	10	6	0		**PIN**	12	0	10	2	0	
Carcinoma	33	0	3	16	14	0.0001*	**Carcinoma**	20	0	1	17	2	0.0001*
Grade 1–2	14	0	3	8	3	0.0293*	**Grade 1–2**	10	0	1	9	0	0.0156*
Grade 3–4	19	0	0	8	11	0.0001*	**Grade 3–4**	10	0	0	8	2	0.0001*

Note: The expression of ROBO1 was evaluated as staining of the tissue as none (0), weak (0.5–1), moderate (1.5–2) and strong (2.5–3). Fisher exact test was used to examine the association between staining intensity and tissue type or staining intensity and tumor grade (for tumor specimen only).

*p< 0.05 was considered significant.