Vav3 oncogene is overexpressed and regulates cell growth and androgen receptor activity in human prostate cancer.

Zhongyun Dong^2, Yin Liu^1, Shan Lu^2, Amy Wang^2, Kiwon Lee^1, Lu-Hai Wang^3, Monica Revelo^1, and Shan Lu^1*

Department of ^1Pathology and ^2Medicine, University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati, OH 45267, USA: ^3Department of Microbiology, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, USA.

Running title: Vav3 oncogene in prostate cancer

Key word: Vav3 oncogene, androgen receptor, prostate cancer, signal transduction

*Corresponding author: Shan Lu, Department of Pathology, University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati, OH 45267, USA, Phone: 513-558-5109, Fax: 513-558-2141, E-mail: shan.lu@uc.edu

Grant supports: This work is supported by Cancer Center Foundation, University of Cincinnati College of Medicine.


Acknowledgement: The authors would like to thank Dr. Zengxin Wang (University of Texas M. D. Anderson Cancer Center, UTMDACC, Houston) and Dr. Anke Klippel (atugen AG, Berlin, Germany) for providing us with plasmids.
ABSTRACT

The purpose of this research was to investigate the role of Vav3 oncogene in human prostate cancer. We found that expression of Vav3 was significantly elevated in androgen-independent LNCaP-AI cells in comparison with that in their androgen-dependent counterparts LNCaP cells. Vav3 expression was also detected in other human prostate cancer cell lines (PC-3, DU145, and 22Rv1) and, by immunohistochemistry analysis, was detected in 32% (26 out of 82) of surgical specimens of human prostate cancer. Knockdown expression of Vav3 by siRNA inhibited growth of both androgen-dependent LNCaP and androgen-independent LNCaP-AI cells. In contrast, overexpression of Vav3 promoted androgen-independent growth of LNCaP cells induced by epidermal growth factor (EGF). Overexpression of Vav3 enhanced androgen receptor (AR) activity regardless of the presence or absence of androgen and stimulated the promoters of AR target genes. These effects of Vav3 could be attenuated by either PI3K inhibitors or dominant-negative Akt and were enhanced by cotransfection of PI3K. Moreover, phosphorylation of Akt was elevated in LNCaP cells overexpressing Vav3, which could be blocked by PI3K inhibitors. Finally, we identified the DH domain of Vav3 was responsible for activation of AR. Taken together, our data show that overexpression of Vav3, through the PI3K-Akt pathway, inappropriately activates AR signaling axis and stimulates cell growth in prostate cancer cells. These findings suggest that Vav3 overexpression may be involved in prostate cancer development and progression.
INTRODUCTION

Compelling data show that the elevated androgen receptor (AR) activity and PI3K-Akt signaling play an important role in prostate cancer development and progression (1-4). The PI3K-Akt pathway is a cell growth and survival pathway for human cancers. Elevated PI3K-Akt signaling, such as mediated by PTEN deletion and mutation, contributes to prostate cancer development and progression. Prostate-specific deletion of murine PTEN, a tumor suppressor gene that shuts off PI3K-Akt signaling, induces the prostatic intraepithelial neoplasia lesions, which later progress to invasive prostate cancer in mice (5). Multiple growth factors and cytokines, including epidermal growth factor (EGF), signal through the PI3K-Akt pathway and inappropriately activates AR in prostate cancer cells, which can be accomplished by direct AR phosphorylation by active Akt (6-8). PI3K-Akt signaling can also induce phosphorylation and inactivation of GSK3β, resulting in an increased nuclear level of β-catenin. Consequently, coactivator β-catenin enhances AR activity to stimulate prostate cancer cell growth and survival (9, 10). Studies from our laboratory as well as others demonstrate that elevated PI3K-Akt signaling is critical for androgen-independent growth of human prostate cancer cells (11). It is noteworthy, however, that an elevation of Akt phosphorylation is observed in 45.8% of prostate cancer specimens (12) and a loss of or reduced PTEN protein expression occurs in approximately 30% of human prostate cancers (13, 14), suggesting the presence of other mechanisms that activate PI3K-Akt signaling in prostate cancer.

Vav gene is an oncogene identified in cell transformation experiments (15). Vav family proteins include three members (Vav1, Vav2, and Vav3) in mammalian cells (16-18). Vav1 is
primarily expressed in hematopoietic lineages, while Vav2 and Vav3 are more ubiquitously expressed (19). Vav proteins share a common structure, including a N-terminal calponin homology (CH) domain involved in Ca\textsuperscript{2+} mobilization and transforming activity, an acidic region containing three regulatory tyrosines, a Dbl homology (DH) domain with a conserved region that promotes the exchange of GDP for GTP on Rac/Rho GTPases, a pleckstrin homology (PH) domain binding to PIP\textsubscript{3} that enables its movement to the inner face of the plasma membrane, two Src-homology 3 (SH3) domains interacting with proteins containing proline-rich sequences, and a Src-homology 2 (SH2) domain interacting with the proteins containing phosphorylated tyrosines (20, 21). Vav proteins function as guanosine nucleotide exchange factors (GEFs) for the Rho/Rac family proteins. Independent of their GEF activity, Vav proteins also work as adapter molecules. Tyrosine phosphorylation by receptor or cytoplasmic protein tyrosine kinase is required for Vav protein activation. In the non-phosphorylation state, the DH domain is masked by the N-terminal extension containing the tyrosine residue Y174. Phosphorylation of Y174 exposes the DH domain, which in turn interacts with substrate proteins (22). Upon ligand stimulation of EGF receptor, Ros, insulin receptor, and IGF receptor, Vav3 is activated and physically associated with a variety of signaling molecules, including Rac1, Cdc42, PI3K, shc, Grb2, and PLC-\textgamma, leading to alteration in cell morphology and cell transformation (23). Similarly, overexpression of Vav3 leads to PI3K activation and focus formation in NIH3T3 cells. Vav3-induced cell transformation can be blocked by overexpression of PTEN or PI3K inhibitor LY294002 (24). However, the role of Vav3 in human cancer has not been described.

The purpose of this study was to determine the potential roles of Vav3 in human prostate cancer. We found that Vav3 was overexpressed in human prostate cancer and in androgen-
independent human prostate cancer cells. Overexpression of Vav3 promoted prostate cancer cell growth and activated AR, regardless of the absence or presence of androgen. The effects of Vav3 on cell growth and AR activity were mediated by activation of the PI3K-Akt pathway. These findings suggest that overexpression of Vav3 inappropriately activates AR and stimulates cell growth, which may play important roles in human prostate cancer development and/or progression.

RESULTS

Vav3 gene expression in prostate cancer cells and human prostate cancer specimens

Previously, we established an androgen-independent cell line LNCaP-AI from its parental androgen-dependent LNCaP cells (25). Taking advantage of the same genetic background between LNCaP and LNCaP-AI cells, we performed oligonucleotide DNA microarray analysis to identify genes that may contribute to androgen-independent growth. One of the most striking findings in this study is overexpression of Vav3 oncogene in LNCaP-AI cells. To evaluate Vav3 protein expression, we generated a rabbit anti-Vav3 antibody against a peptide located at the C-terminus of Vav3, which is specific for Vav3 of both human and mouse origin and does not cross with Vav1 and Vav2. As shown in Fig. 1A, the 98 kD Vav3 was overexpressed in LNCaP-AI cells as compared with that in LNCaP cells. Similarly, Vav3 protein was detected in three other androgen-independent human prostate cancer cell lines (PC3, DU145, and 22RV1) (Fig. 1A).
To determine the potential role of Vav3 in prostate cancer, we evaluated Vav3 expression in surgical specimens of human prostate cancer by immunohistochemistry (IHC). Eighty-two tumor tissues and 10 normal tissues from patients of 38-87 year-old were analyzed (Table 1). Vav3 protein was detected in 26 tumor tissues (32%). All normal prostate tissues showed negative immunoreactivity for Vav3. As shown in Fig. 1B, Vav3 protein was located in the tumor epithelium but not stroma or normal prostate tissues. A significance difference was found for Vav3 gene positive (n=26) and negative (n=56) prostate cancer specimens (P < 0.001) using binomial method and log link function. A retrospective power analysis was carried out to determine the association of Vav3 expression and Gleason score and showed that the study had approximately 52% power to detect a significant trend given the observed data, assuming a significance level of alpha=5%. Therefore, our sample size is not large enough to demonstrate the association between Vav3 expression and Gleason score in prostate cancer.

**Vav3 is involved in growth of prostate cancer cells**

To determine the role of Vav3 in growth of prostate cancer cells, we used siRNA technology to reduce Vav3 gene expression. We designed a siVav3-247 oligonucleotide specific for Vav3 gene and control-247 oligonucleotide. After transfection with different concentrations of siVav3-247 or control-247, LNCaP cells were cultured in stripped medium supplemented with 10^-8M of dehydrotestosterone (DHT) and LNCaP-AI cells were cultured in stripped medium in 96-well plate. As shown in Fig. 2A, transfection of siVav3-247, but not control-247, dose-dependently inhibited both androgen-dependent growth in LNCaP cells and androgen-independent growth in LNCaP-AI cells. At 1.0 pm/well of siRNA concentration, transfection of siVav3-247 inhibited
growth of LNCaP and LNCaP-AI cells by 53% and 65%, respectively. In contrast, transfection of the same amount of control-247 resulted in 15% inhibition in LNCaP cells and 18% in LNCaP-AI cells. Knockdown expression of Vav3 by siVav3-247 was confirmed at the protein level (Fig. 2B). Densitometry analysis of Vav3 relative to β-actin showed that the Vav3 levels decreased by 80% and 45% on day 3 and day 5, respectively, in LNCaP cells transfected with siVav3-247. In contrast, transfection of control-247 only reduced Vav3 by 22% and 1% in LNCaP cells on 3 day and 5 day, respectively.

We then determined whether overexpression of Vav3 in LNCaP cells could stimulate cell growth. LNCaP cells were stably transfected with wild type Vav3, constitutive active Vav3*, or empty vector pHEF to derive LNCaP-Vav3, LNCaP-Vav3*, and LNCaP-HEF cell lines, respectively. Overexpression of 98 kD Vav3 and 75 kD Vav3* was confirmed at the protein level (Fig. 3A). Overexpression of Vav3 or Vav3* did not alter AR expression in these cells (Fig. 3B). We found that overexpression of constitutive active Vav3* significantly stimulated cell growth in the absence of androgen. On day 6, the OD reading, which reflects the cell density, in wells containing LNCaP-Vav3* cells, was 1.6 fold higher than those containing LNCaP-HEF cells (Fig. C). Overexpression of wild type Vav3 potentiated growth stimulatory effects of EGF (Fig. 3D). In the cultures containing 1.6 ng/ml of EGF, the density of LNCaP-Vav3 cells was 3 fold higher than that of LNCaP-HEF cells. Overexpression of Vav3 or Vav3* did not significantly stimulate LNCaP cell growth in the presence of DHT in MTT assay, which may be that the robust DHT effect overshadowed Vav3-mediated cell growth in the short term of cell growth experiment. Therefore, our data suggest that overexpression of Vav3 promoted androgen-independent growth in prostate cancer cells.
To determine the role of Vav3 in activation of AR, we investigated whether overexpression of Vav3 could regulate AR-dependent luciferase reporter activities. Hela cells were transiently cotransfected with an ARE-driven luciferase reporter without or with an AR expression vector, as well as Vav3 or Vav3*. As shown in Fig. 4A, minimal luciferase activity was detected in cells without cotransfection of AR, regardless of stimulation by DHT or transfection of Vav3 or Vav3*. Expression of AR moderately increased basal level of the ARE-driven luciferase activity, which was elevated in cells treated with DHT. Similarly, this AR-dependent luciferase activity was enhanced by approximately 1.9 and 3.5 fold in cells overexpressed with Vav3 and Vav3*, respectively (Fig. 4A). These data indicate that the elevated ARE promoter activity in Hela cells was mediated by AR. Furthermore, Vav3 dramatically enhanced AR activity at the below physiological level of androgen (Fig. 4B). The effects of Vav3 on AR activation were verified in LNCaP-AI cells, which had higher and more consistent transfection efficiency in transient transfection experiments. Data in Fig. 4C show that overexpression of Vav3 enhanced AR activity in both presence and absence of its ligand DHT in LNCaP-AI cells. In agreement with its effects on androgen-independent growth of cells overexpressing Vav3 (Fig. 3D), overexpression of Vav3 potentiated EGF-stimulated AR activity in LNCaP-AI cells (Fig. 4D). On the other hand, we determined whether blocking AR activity compromised Vav3-mediated AR activation. We found that AR antagonist Flutamide inhibited AR activation mediated by both DHT and Vav3 (Fig. 4E).
PI3K-Akt signaling enhances AR activity and is thought to be critical for prostate cancer development and progression (6, 11). Since Vav3 is a potential upstream regulator of the PI3K-Akt pathway (23, 24), we investigated whether overexpression of Vav3 could affect PI3K-Akt signaling-mediated AR activation. Hela cells were transiently cotransfected with ARE-Luc reporter, AR expression vector, Vav3*, and p85 (the PI3K regulatory subunit) and/or p110 (the catalytic subunit). As shown in Fig. 5A, transfection of Vav3* or either subunits of PI3K stimulated AR activity. AR activity was further enhanced in cells cotransfected with both Vav3* and either subunits of PI3K. To determine the role of endogenous PI3K in Vav3-induced AR activation, we investigated effects of PI3K inhibitors LY294002 and Wortmannin on AR activation. After transfection of LNCaP-AI cells with luciferase reporters driven by ARE or the promoters of PSA or p21WAF/CIP genes, both are AR-responsive genes (26, 27), and Vav3 or Vav3*, the cells were treated for 24 hour with 5 µM of LY294002 or 0.5 µM of Wortmannin. PI3K inhibitors blocked both basal and Vav3- or Vav3*-stimulated activation of ARE promoter in the absence (Fig. 5B) and presence (Fig. 5C) of DHT. The PI3K inhibitors also blocked Vav3-mediated activation of PSA (Fig. 5D) and p21WAF/CIP (Fig. 5E) promoters. Consistently, the basal and Vav3*-stimulated p21WAF/CIP promoter activity was inhibited by cotransfection of dominant-negative Akt (Fig. 5F). These data suggest that Vav3 activates AR, which is mediated by PI3K-Akt signaling.

We directly investigated effects of Vav3 and PI3K-Akt signaling on expression of androgen-responsive p21WAF/CIP gene at protein level by western blot analysis. LNCaP cells stably transfected with Vav3 expressed higher level of Vav3 (Fig. 3A) and p21WAF/CIP (Fig. 6). Treatment with DHT (10^-8 M) enhanced p21WAF/CIP expression in control (LNCaP-HEF) and
Vav3-engineered LNCaP-Vav3 cells (Fig. 6A). Moreover, although there was no discernable difference in Akt expression levels, phosphorylated Akt was significantly elevated in LNCaP-Vav3 cells relative to that in LNCaP-HEF cells (Fig. 6B). Treatment with LY294002 reduced the levels of phosphorylated Akt in both LNCaP-HEF and LNCaP-Vav3 cells. These data indicated that overexpression of Vav3 enhanced AR-target gene expression, which was mediated by activation of PI3K-Akt signaling.

The DH domain of Vav3 is responsible for activation of AR

To dissect the functional domain of Vav3 involved in AR activation, we generated truncated constructs of Vav3* with either deletion of the DH domain (Vav3*-ΔDH) that has GEF activity or the SH domain that has adaptor activity (Vav3*-ΔSH) (Fig. 7A). We found that the ARE promoter activity was moderately enhanced in cells cotransfected with Vav3 and was significantly elevated in cells cotransfected with Vav3* in Hela cells (Fig. 7B). Deletion of the DH domain of Vav3* abolished its activity in activation of ARE promoter. In sharp contrast, the removal of the SH domain did not alter the effects of Vav3* on ARE promoter activation (Fig. 7B). This experiment was also confirmed in LNCaP cells without cotransfection of AR expression vector. Since Vav3*-ΔDH construct still contains the PH domain with PIP3 binding activity, these data also suggest that binding of PIP3 does not play an essential role for AR activation by Vav3*. However, our data did not exclude the regulatory role of the PH domain for Vav3 activity in AR activation. This finding indicates that the DH domain was responsible for activation of AR.
DISCUSSION

We have investigated the potential roles of Vav3 in human prostate cancer. We found that Vav3 protein was detected in all human prostate cancer cell lines examined. The expression of Vav3 was further elevated in androgen-independent human prostate cancer cells in comparison with their androgen-dependent counterpart. Moreover, Vav3 protein was detected approximately 30% of human prostate cancer tissues. Downregulation of Vav3 inhibited both androgen-dependent and androgen-independent growth of human prostate cancer cells. In sharp contrast, overexpression of Vav3 significantly enhanced EGF-stimulated androgen-independent growth. These data suggest that overexpression of Vav3 is involved in prostate cancer cell growth and that Vav3 may play an important role in human prostate cancer development and progression to androgen-independent growth.

AR is expressed in most prostate cancers and elevated AR activity is thought to be involved in prostate cancer development and progression (28, 29). AR mutation and gene amplification are common alterations in advanced human prostate cancer and associated with the disease progression (30, 31). Mutations in the ligand binding domain generate promiscuous AR, which accepts a broad ligand spectrum for activation, such as by unfavorable adrenal androgen and AR antagonists (32, 33). Overexpression of steroid hormone receptor coactivators, such as SRC1, SRC3, TIF2, and ARA55, dramatically enhances AR activity (34-36). However, probably more importantly, AR can be activated by phosphorylation (37, 38). For instance, activation of PI3K-Akt signaling upregulates AR activity (3, 6, 11). Data presented in this paper reveal that
overexpression of Vav3 could be a novel mechanism that inappropriately activates AR in human prostate cancer cells. This effect is mediated by PI3K-Akt signaling. Four lines of evidence support this conclusion. First, overexpression of Vav3 activates both liganded- and unliganded-AR in prostate cancer cells, which can be inhibited by Flutamide. Vav3 dramatically enhances AR activity by androgen at concentrations of below the physiological level. Second, overexpression of Vav3 enhances promoter activities of AR-target genes PSA and p21WAF/CIP, which can be attenuated by PI3K inhibitors or dominant-negative Akt. On the other hand, AR activity is enhanced by cotransfection of Vav3 and PI3K. Third, overexpression of Vav3 leads to upregulation of endogenous p21WAF/CIP expression, which is further enhanced by DHT. Constitutive phosphorylation of Akt is elevated in LNCaP cells overexpressing Vav3, which can be blocked by PI3K inhibitors. Finally, the DH domain of Vav3 is identified to be responsible for regulation of AR activity. Moreover, we found that overexpression of Vav3 potentiates EGF-stimulated AR activation and prostate cancer cell growth. Previous studies have demonstrated that EGF stimulates prostate cancer cell growth, which is inhibited by AR antagonists Casodex and ICI 182780, suggesting an AR-mediated mechanism (39, 40).

During preparation of this manuscript, one most recent publication also showed that Vav3 upregulates AR activity and supports androgen-independent growth in prostate cancer cells by Lyons and Burnstein (41). However, in contrast to our finding that AR activation by Vav3 is mediated by PI3K-Akt signaling in LNCaP cells, they found that effects of Vav3 in PC3 cells were not inhibited by PI3K inhibitor. Since two different cell lines were used in these studies, the discrepancy could be due to the heterogeneity of prostate cancer cell lines. We identified that the DH domain of Vav3 is responsible for AR activation by deletion mutation analysis, which is an
approach by taking advantage of multiple distinct function domains of Vav3. We found that deletion of the DH domain, but not the PH domain, abolished the Vav3-mediated AR activation. Our data did not exclude the regulatory effect of the PH domain, a PIP3 binding site, for Vav3 activity, which may be what was observed by Lyons and Burstein’s study. The DH domain of Vav3 contains GEF activity for Rho/Rac small G proteins. We investigated whether these small G proteins are involved in AR activation. Consistently, we also found that neither Rac1 nor RhoA, both constitutive active or dominant negative forms, showed impact on Vav3-mediated AR activation.

Recently, we reported that signaling level of the PI3K-Akt pathway is elevated in LNCaP-AI cells relative to that in LNCaP cells and is critical for their androgen-independent growth (11). This elevated PI3K-Akt signaling in LNCaP-AI cells is not due to PTEN, because PTEN is mutated in LNCaP cells (4). Data presented in this paper show that overexpression of Vav3 is at least partially responsible for the further elevation of PI3K-Akt signaling in LNCaP-AI cells. Our finding implicates that Vav3 overexpression is an alternative mechanism that leads to elevated PI3K-Akt signaling and AR activity in human prostate cancer, which may be of great importance in prostate cancer development and progression. Further studies are necessary to determine whether Vav3 activates AR by direct binding, enhances nuclear localization, and/or AR chromosome occupancy. We will also address whether Vav3 overexpression and loss of or reduced PTEN protein expression occur additively or mutual-exclusively in human prostate cancer and whether overexpression of Vav3 in prostate cancer and further elevation in androgen-independent cells, e.g. LNCaP-AI vs. LNCaP cells, are due to Vav3 gene amplification or an upregulation of Vav3 gene transcription.
In summary, this study revealed that Vav3 is overexpressed in human prostate cancer specimens and is further elevated in androgen-independent prostate cancer cells. Vav3 overexpression, independent of PTEN, is involved in aberrant cell growth, AR activation, and induction of AR target genes in human prostate cancer cells. These effects were mediated by PI3K-Akt signaling. Therefore, overexpression of Vav3 could be a new marker for human prostate cancer. Targeting Vav3 could be an efficacious therapeutic modality for prostate cancers, especially those androgen-independent diseases.

MATERIALS AND METHODS

Reagents

RPMI 1640 medium was purchased from Invitrogen (Gaithersburg, MD). Fetal bovine serum (FBS) and charcoal/dextran-treated FBS were purchased from HyClone Laboratories (Logan, UT). Antibodies against Akt and phospho-Akt were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti-p21 antibody was obtained from Oncogene Research Products (La Jolla, CA). Human Vav3-specific Stealth™ Select RNAi (siVav3-247: 5’-CCCAGTTTCTCTGTTTGAAGACAT-3’) and its control (control-247: 5’-CCCTTCTCTGTTTTGAAAGACAT-3’) were designed by a software in Invitrogen website and we purchased both oligos from Invitrogen. The transfection reagent Lipofectamine™ 2000 were purchased from Invitrogen.
Anti-Vav3 antibody from Upstate Biotechnology (Charlottesville, VA) was used for IHC analysis. We also generated our custom anti-Vav3 antibody from the C-terminus of Vav3 peptide sequence (CMELVEYYKHSLKEGFRT). We determined Vav3 expression from normal mouse prostate tissues and LNCaP cells and no non-specific band was detected by this antibody. Wild type Vav3 showed a 98 kD band and Vav3* showed a 75 kD band. Our custom Vav3 antibody was used for western blot analysis in this study.

**Cell culture**

The human prostate adenocarcinoma cell lines (LNCaP, PC-3, DU145, and 22Rv1) and cervical carcinoma cell line Hela were obtained from ATCC (Rockville, MD) and maintained in RPMI-1640 medium supplemented with 10% FBS (complete medium) at 37°C in 5% CO₂. The androgen-independent LNCaP-AI cells were derived from LNCaP cells by long-term culture of the latter in RPMI-1640 medium supplemented with 10% charcoal/dextran-treated FBS (stripped medium) (25). LNCaP-AI cells were maintained in stripped medium.

**Plasmids**

The construction and structure of pBEF-Vav3, pHEF-Vav3*, and control vector pHEF were detailed in our previous studies (24). Vav3* is a constitutive active form of Vav3 due to the CH domain and acidic region deletion at N-terminus (Fig. 7A). PSA(-4.3)-Luc and pGL3-ARE-E4-Luc, in which luciferase reporter gene is driven by a 4.3 kb PSA promoter and 4 copies of androgen response element (ARE) fused with adenovirus E4 minimal promoter at the upstream,
respectively, were generously provided by Dr. Zhengxin Wang. The p21(-215)-Luc reporter containing 215 base pair of p21^{WAF/CIP} promoter sequence including ARE detailed in our previous studies (26, 27). The PI3K expression vectors were gifts from Dr. Anke Klippel, atugen AG, Berlin, Germany.

Vav3*-ΔSH was generated by deletion SH2 and SH3 fragment at the C-terminus of Vav3* using Bam H1 site and subsequently a linker (5’ GATCCATAATGATGC3’) containing stop codon was inserted in frame. Vav3*-ΔDH was generated by PCR amplification of PH domain with an upstream primer (5’ CGAGAATTCGATGGAGCGTGGGAAGCAG3’) containing a translation start codon ATG in frame and downstream primer (5’ CCTGGATCCACCTGTTTAGGA3”) containing Bam H1 site, and this PCR fragment was inserted at Bam H1 site of Vav3* sequence with the DH an PH domain deletion using the Bam H1 site.

**Cell growth assay**

Tumor cell growth was estimated by the MTT assay as previously described (25). Briefly, LNCaP and LNCaP-AI cells were seeded into 96-well cell culture plates at a density of 5x10^3 cells/well in stripped medium. After incubation in 5% CO_2 at 37°C overnight, the cells were treated with various concentration of drugs in stripped medium for 5 days. At the end of incubation, 20 ul of MTT (2.5 mg/ml in PBS) was added to each well, and the cells were further incubated for one hour at 37°C to allow complete reaction between the dye and the enzyme mitochondrial dehydrogenase in the viable cells. After removal of the residual dye and medium,
100 ul of dimethylsulfoxide was added to each well, and the absorbance at 570 nm was measured using BMG microplate Reader (BMG Labtech, Inc., Durham, NC).

**Western blot analysis**

Western blot analysis was performed as previously described (25). Briefly, aliquots of samples with the same amount of protein, determined using the Bradford assay (BioRad, Hercules, CA), were mixed with loading buffer (final concentrations of 62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 100 mM dithiothreitol, and 0.005% bromphenol blue), boiled, fractionated in a SDS-PAGE, and transferred onto a 0.45-um nitrocellulose membrane (BioRad). The filters were blocked with 2% fat-free milk in PBS, and probed with first antibody in PBS containing 0.1% Tween 20 (PBST) and 1% fat-free milk. The membranes were then washed four times in PBST and incubated with horseradish peroxidase-conjugated secondary antibody (BioRad) in PBST containing 1% fat-free milk. After washing four times in PBST, the membranes were visualized using the ECL Western blotting detection system (Amersham Co., Arlington Height, IL). For western blot analysis of Vav3 expression, milk-free TBS buffer (pH 8.0) was used for the first antibody overnight at 4°C.

**Transient transfection assay**

Cells (10^5/well) were seeded in 12 well tissue culture plates. Next day, Optifect-mediated transfection was used for the transient transfection assay according to the protocol provided by Invitrogen/Life Technologies, Inc. The cells were then treated with hormone or drugs in stripped
medium for 24 hours. Subsequently, the cell extracts were prepared and luciferase activity was assessed in a Berthold Detection System (Pforzheim, Germany) using a kit (Promega, Madison, WI) following the manufacture’s instruction. For each assay, cell extract (20 ul) was used and the reaction was started by injection of 50 ul of luciferase substrate. Each reaction was measured for 10 seconds in the Luminometer. Luciferase activity was defined as light units/mg protein.

**Immunohistochemistry (IHC) analysis of Vav3 expression**

The paraffin-embedded tissue sections of human prostate cancer specimens for immunohistochemistry analysis are from our prostate cancer tissue bank (Department of Pathology, University of Cincinnati College of Medicine) and prostate cancer tissue array obtained from US Biomax Inc. (Rockville, MD).

The paraffin-embedded tissue sections or tissue arrays were deparaffinized in xylene, rehydrated in graded alcohol, and transferred to PBS. The slides were treated in a microwave oven (1500 Watts) at 80% power output for 2 x 5 min in 0.1 M citric acid to retrieve antigens, followed by a treatment with 3% H₂O₂ in methanol (v/v) to block endogenous peroxidase. The treated slides were incubated in blocking buffer (5% BSA) at room temperature for 1 hour and then with the blocking buffer containing 1~2 ug/ml of rabbit anti-Vav3 antibody (Upstate Biotechnology) or rabbit IgG (Santa Cruz Biotechnology) overnight at 4°C. After washing, the slides were incubated with a biotinylated secondary antibody (from BioGenex Laboratories, San Ramon, CA), followed by washing and incubation with the streptavidin conjugated peroxidase (BioGenex). A positive reaction was visualized by incubating the slides with stable
diaminobenzidine and counterstaining with Gill’s hematoxylin (BioGenex). After washing three times with PBS, the slides were mounted with Universal Mount mounting medium (Fisher Scientific). The intensity and extent of cytoplasm positive labeling for Vav3 antibody were assessed semi-quantitatively and scored as 0 (no staining), 1+ (weak and focal staining in less than 25% of tissue), 2+ (moderate intensity in 25 ~ 50% of tissue), and 3+ (strong and diffused staining in more than 50% of tissue).

REFERENCES


neoplastic prostate tissues are strong predictors of biochemical recurrence. Clin Cancer Res 10:6572-6578


41. Lyons LS, Burnstein KL 2005 Vav3, a Rho GTPase Guanine Nucleotide Exchange Factor, Increases During Progression to Androgen Independence in Prostate Cancer Cells and Potentiates Androgen Receptor Transcriptional Activity. Mol Endocrinol
Figure legend

Figure 1. Expression analysis of Vav3 in prostate cancer cells and in human prostate cancer specimens.

(A) Vav3 expression in prostate cancer cell lines. 100 ug of cell extracts was used for Western blot analysis of Vav3 expression in LNCaP and LNCaP-AI cells, as well as PC3, DU145, and 22RV1 cells. β-actin was served as loading control. (B) IHC analysis of Vav3 expression in human prostate cancer specimens. (I) The normal prostate tissues are shown an area of unremarkable prostate glands and stroma with negative immunoreactivity for Vav3. (II) An area of prostatic adenocarcinoma Gleason score 6 (3+3) with moderate to intense diffuse immunoreactivity for Vav3 (AII). An area of prostatic adenocarcinoma Gleason score 8 (4+4) with intense diffuse immunoreactivity for Vav3. (III) The microphotographs of immunohistochemical staining are shown at original magnification of 400X.

Figure 2. Knockdown expression of Vav3 inhibits cell growth.

(A) LNCaP and LNCaP-AI cells (5000/well in 96-well plate) were transiently transfected with siVav3-247 or control-247 at the concentrations of 0, 0.25, 0.5, and 1.0 pM/well using Lipofectamine 2000 for overnight. Then, LNCaP cells were cultured in stripped medium with 10^-8 M of DHT and LNCaP-AI cells were cultured in stripped medium for 5 days, followed by MTT assay. The data was presented as % inhibition. (B) Expression analysis of Vav3 upon Vav3 siRNA treatment. LNCaP cells were transfected with 5 pM/well of siVav3-247 or control-247 in
6-well plate for 3 and 5 days. The cell extracts were prepared and subjected to western blot analysis for Vav3. β-actin was served as loading control.

Figure 3. The role of Vav3 in prostate cancer cell growth.

(A and B) Vav3 expression in LNCaP-Vav3, LNCaP-Vav3*, and LNCaP-pHEF cell lines. 100 ug of cell extracts from each cell line was used for Western blot analysis of Vav3 and AR expression. β-actin was served as loading control. (C) LNCaP-HEF or LNCaP-Vav3* cells (5,000 cells per well) were seeded into 96 well plate in stripped medium. MTT staining for one hour was performed on days 0, 1, 3, and 6. (D) LNCaP-HEF and LNCaP-Vav3 cells were cultured in stripped medium in the presence of various concentration of EGF for 5 days, followed by MTT assay. The error bar represents the average of triplicate samples of each experiment.

Figure 4. Vav3 is involved in AR activation.

(A and B) Hela cells (10^5 cells/well in 12-well plate) were cotransfected with pGL3-ARE-E4-Luc (0.25 ug), expression vector (100 ng) for Vav3, Vav3*, or empty vector pHEF, as well as expression vector (25 ng) for AR, or empty vector pCMV, respectively. Then, the cells were treated with DHT (10^{-8}M) (A) or 10^{-12}M and 10^{-11}M of DHT (B). (C and D) LNCaP-AI cells (2X10^5 cells/well in 12-well plate) were cotransfected with pGL3-ARE-E4-Luc (1 ug), expression vector (0.5 ug) for Vav3 or empty vector pHEF, respectively. Then, the cells were treated with DHT (10^{-8} M) (C) or EGF (10 ng/ml) (D). (E) LNCaP-AI cells were cotransfected with pGL3-ARE-E4-Luc (1 ug) and expression vector for Vav3* (0.5 ug). Then, the cells were treated with Flutamide (10 uM) without or with DHT (10^{-10}M). All transfection and drug
treatment are in stripped medium for 24 hours, followed by luciferase assay. Renilla luciferase as an internal control was used to normalize the data. Data are presented as the mean (±SD) of duplicate values of a representative experiment that was independently repeated for five times.

**Figure 5. Vav3 activates AR via the PI3K-Akt pathway.**

(A) Hela cells were cotransfected with pGL3-ARE-E4-Luc (0.25 ug), expression vector (50 ng) for Vav3, Vav3*, or empty vector pHEF, and expression vector (50 ng) for p85, p110, p85+p110, or empty vector pCMV, as well as expression vector (25 ng) for AR, or empty vector pCMV, respectively. (B and C) LNCaP-AI cells were cotransfected with pGL3-ARE-E4-Luc (1 ug) and expression vector for Vav3* (0.5 ug). Then, the cells were treated with LY294002 (10 uM) or Wortmannin (0.5 uM) without (B) or with DHT (C). (D and E) LNCaP-AI cells were cotransfected with PSA(-4.3)-Luc (1 ug) (D) or p21(-215)-Luc (1 ug) (E), as well as expression vector (0.5 ug) for Vav3, Vav3*, or empty vector pHEF, respectively. Then, the cells were treated with LY294002 or Wortmannin. (F) LNCaP–AI cells were cotransfected with p21(-215)-Luc reporter (1 ug/well in 12-well plate), dominant-negative Akt (0.25 ug) or empty vector pUS, as well as Vav3* expression vector (0.25 ug) or empty vector pHEF, respectively in stripped medium. All transfection and drug treatment are in stripped medium for 24 hours, followed by luciferase assay. Renilla luciferase as an internal control was used to normalize the data. Data are presented as the mean (±SD) of duplicate values of a representative experiment that was independently repeated for five times.

**Figure 6. Effects of Vav3 on p21<sub>WAF/CIP</sub> expression and Akt phosphorylation.**
(A) LNCaP-HEF and LNCaP-Vav3 cells were treated with DHT (10⁻⁸M) in stripped medium for 24 hours, followed by western blot analysis for p21\textsuperscript{WAF/CIP}. (B) LNCaP-HEF and LNCaP-Vav3 cells were treated with LY294002 in stripped medium for 24 hours, followed by western blot analysis for p21\textsuperscript{WAF/CIP}, Akt, and Phospho-Akt. β-actin was served as loading control.

**Figure 7. Determination of the functional domain of Vav3 involved in AR activation.**

(A) Deletion constructs of Vav3 gene. (B) Hela cells (10⁵ cells/well in 12-well plate) were cotransfected pGL3-ARE-E4-Luc (0.25 ug) with expression vector (100 ng) for Vav3, Vav3*, Vav3*-ΔDH, Vav3*-ΔSH, or empty vector pHEF, as well as expression vector (25 ng) AR for 24 hours, respectively, followed by luciferase assay. Renilla luciferase as an internal control was used to normalize the data. Data are presented as the mean (±SD) of duplicate values of a representative experiment that was independently repeated for five times.
Table 1: Summary of IHC analysis of Vav3 expression in prostate cancer specimens based on Gleason score.

<table>
<thead>
<tr>
<th>Gleason score</th>
<th>Case#</th>
<th>Vav3 positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>18</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>26</td>
<td>7</td>
</tr>
<tr>
<td>9</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>Total prostate cancer specimens</td>
<td>82</td>
<td>26</td>
</tr>
<tr>
<td>Normal prostate tissues</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>
Fig. 1. Dong et al.
Fig. 2. Dong et al.
Fig. 3. Dong et al.
Fig. 4. Dong et al.
Fig. 5. Dong et al.
A. | LNCaP-HEF | LNCaP-Vav3 |
---|---|---|
DHT - | + | - | + | (10^{-8}M) |
p21 | | |
β-actin | | |

B. | LNCaP-HEF | LNCaP-Vav3 |
---|---|---|
LY294002 - | + | - | + | (10 \text{ uM}) |
p21 | | |
P-Akt | | |
Akt | | |
β-actin | | |

Fig. 6. Dong et al.
Fig. 7. Dong et al.