Vav3-Rac1 Signaling Regulates Prostate Cancer Metastasis with Elevated Vav3 Expression Correlating with Prostate Cancer Progression and Posttreatment Recurrence

Kai-Ti Lin¹, Jianli Gong², Chien-Feng Li³, Te-Hsuan Jang¹, Wen-Ling Chen¹, Huei-Jane Chen¹, and Lu-Hai Wang¹,³

Abstract
Prostate cancer remains the second leading cause of cancer death in men in the Western world. Yet current therapies do not significantly improve the long-term survival of patients with distant metastasis. In this study, we investigated the role of the guanine nucleotide exchange factor Vav3 in prostate cancer progression and metastasis and found that Vav3 expression correlated positively with prostate cancer cell migration and invasion. Stimulation of the receptor tyrosine kinase EphA2 by ephrinA1 resulted in recruitment and tyrosine phosphorylation of Vav3, leading to Rac1 activation as well as increased migration and invasion in vitro. Reduction of Vav3 resulted in fewer para-aortic lymph nodes and bone metastasis in vivo. Clinically, expression of Vav3 and EphA2 was elevated in late-stage and metastatic prostate cancers. Among patients with stage IIB or earlier prostate cancer, higher Vav3 expression correlated with lower cumulative biochemical failure-free survival, suggesting that Vav3 may represent a prognostic marker for posttreatment recurrence of prostate cancer. Together, our findings provide evidence that the Vav3-mediated signaling pathway may serve as a therapeutic target for prostate cancer metastasis.

Introduction
The Vav3 oncogene, the third member of the Vav family of Rho GTPase nucleotide exchange factors (GEF; refs. 1–3), is involved in various cellular signaling processes, acting through its classical Dbl domain to activate the Rho family GTPases, including RhoA, Rac1, and Cdc42 (3, 4). Various receptor tyrosine kinases (RTK) activate Vav proteins (3, 5), resulting in the opening up of the Dbl domain for its substrate (6). Deficiency of Vav3 leads to increased bone mass density (7), sympathetic hyperactivity, cardiovascular dysfunction (8), and impaired wound healing (9).

The Eph receptors are the largest family of RTKs and have significant roles in the regulation of cell attachment, cell shape, and motility during development and pathologic conditions, especially cancer metastases (10–12). One Eph receptor, EphA2, is overexpressed in human prostate (13, 14) and other cancers (15). The EphA2 receptor is required for the regulation of cell motility, cell invasion, and in vivo metastasis in prostate cancer (16) and it directly activates Vav2/3 in the regulation of ephrinA1-induced angiogenesis (17). It is, therefore, a good candidate for an upstream mediator of Vav3 activation and subsequent signaling in prostate cancer progression.

Previous studies have extensively investigated the biochemical functions of Vav3; however, its role in tumorigenesis remains unclear. Vav3 expression is increased in androgen refractile prostate cancer cell lines and in prostate tumors (18–20). Overexpression of Vav3 promoted prostate cancer cell growth and enhanced the transcriptional activity of the androgen receptor (refs. 21, 22). Dong and colleagues further described the involvement of Vav3 in the regulation of secretory phospholipase A2-IIa expression in prostate cancer (23). In the study of Liu and colleagues, mice with targeted Vav3 overexpression in the prostate epithelium developed nonbacterial chronic prostatitis in the prostate gland, which was associated with increased incidence of prostate cancer (24).

The involvement of Vav3 in the regulation of cytoskeletal reorganization and increased Vav3 expression in prostate cancer (18, 19) suggests that Vav3 has a role in prostate cancer metastasis. Herein, the present study shows Vav3 involvement in regulation of migration/invasion in prostate cancer cells, mainly through the RTK-activated Vav3-Rac1 signaling axis. In vivo analyses show that Vav3 knockdown in PC3 cells results in significantly lower incidence of lymph node and bone metastasis. Clinical analyses further suggest the prognostic potential of Vav3 in early detection of posttreatment recurrence in prostate cancer patients. Overall, the findings of this

Authors’ Affiliations: ¹Institute of Molecular and Genomic Medicine, National Health Research Institutes, Miaoli County, Taiwan; ²Department of Pharmacology, College of Physicians and Surgeons, Columbia University; ³Department of Microbiology, Mount Sinai School of Medicine, New York; and ⁴Department of Pathology, Chi-Mei Foundation Medical Center, Tainan, Taiwan

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Lu-Hai Wang, National Health Research Institutes, 35 Keyan Road, Zhunan Town, Miaoli County 35053, Taiwan. Phone: 886-37-246166 ex. 35300; Fax: 886-37-585-242; E-mail: lu-hai.wang@nhri.org.tw

doi: 10.1158/0008-5472.CAN-11-2502
©2012 American Association for Cancer Research.

www.aacrjournals.org
study suggest the importance of the Vav3-Rac1 signaling pathway in prostate tumor progression and metastasis.

Materials and Methods

Vectors, antibodies, and reagents

Plasmids including pHEF Vav3, Vav3-(6-10), dominant-negative (dn) RhoA, dnCdc42, dnRac1 or constitutively active (ca) Rac1, and GST-PK CRIB have been described previously (3, 25). The PCDNA-EphA2 plasmid was amplified from PC3 cells and cloned into PCDNA3. Antibody information is provided in the Supplementary Material.

RNA interference and short hairpin RNA construction

Three Vav3-specific siRNAs were obtained from Invitrogen. The target sequences are provided in Supplementary Table S2. The sequences of the Vav3-specific short hairpin RNAs (shRNA) are 5'-GGAAGGGTTCAAGACCTTA-3' and 5'-GAA-GATCTCTATGACTGTTG-3'. These sense and antisense plus hairpin sequences were cloned into pSuper Vector (Oligoengine).

Cell culture, transfections, and stable cell line generation

The LNCaP and PC3 cell lines were obtained from BCRC (Hsinchu, Taiwan); C4-2 was a gift from Dr. Simon Hall of Mount Sinai School of Medicine (New York, NY). The LNCaP and C4-2 cells were maintained in RPMI medium (Invitrogen) with 10% FBS (Biological Industries). The PC3 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen) with 10% FBS (Biological Industries). The PC3 cells were transfected using Lipofectamine 2000 (Invitrogen). The PC3 cell lines stably expressing pSuper-shControl and pSuper-shVav3 were established by selection with Puromycin (Sigma-Aldrich). The stable clones used were combined from 3 single clones selected from control or Vav3 shRNA–expressing cells.

RNA purification and real-time reverse transcriptase PCR

Illustra RNAspin Mini Kits (GE Healthcare Life Sciences) were used to extract RNA, following protocols supplied by the manufacturer. First-strand cDNA was generated using the ReverTra Ace (TOYOBO). Real-time reverse transcriptase PCR (RT)-PCR was carried out on a 1:10 dilution of cDNA, using KAPA SYBR FAST qPCR Kits (KAPA Biosystems) and a CFX96 real-time PCR detection system (Bio-Rad). The mRNA levels were then normalized to actin mRNA. All the primer sequences used in this study are provided in Supplementary Table S2. All experiments were repeated 2 times.

Immunoprecipitation and Western blot analysis

To activate EphA2, serum-starved cells were stimulated with 2 μg/mL preclustered ephrin-A1-Fc (R&D Systems) for 10 minutes. The ephrinA1-Fc or control Fc (Jackson Immunoresearch) chimeras were preclustered by incubating with anti-human IgG (Jackson Immunoresearch), in the ratio 1:2, at 4°C for 1 hour before stimulation. Cells were lysed in lysis buffer (see Supplementary Material). The cell lysates were incubated with antibody against Vav3, together with protein G-sepharose beads for 2 hours at 4°C. After 3 washes, the bound proteins were eluted and analyzed using Western blotting. Protein levels were quantified using densitometry and normalized to Vav3, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), or actin levels.

Assay of Rac1 activation

The glutathione–sepharose beads conjugated with GST-PK CRIB were purified and used as described (3). Cell lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer (see Supplementary Material). The GST-PK CRIB–conjugated beads were used to pull-down GFP-Rac1 from the cell lysates for 45 minutes at 4°C. The beads were washed 3 times. Bound proteins were eluted and analyzed by Western blotting with anti-Rac1. The levels of GFP-Rac1 were quantified using densitometry and normalized to total Rac1 levels.

Cell migration and cell invasion assay

Cell migration and invasion were assayed in 8.0-μm Falcon Cell Culture Inserts with or without Matrigel (BD Biosciences; ref. 25). For cells receiving ephrinA1 stimulation, 0.5 × 10⁵ to 2 × 10⁵ cells were starved overnight, suspended in DMEM (300 μL), and plated in the 0.3-cm² upper Transwell chamber. The bottom well was filled with 500 μL DMEM with 2 μg/mL preclustered ephrin-A1-Fc or control Fc. For cells receiving serum stimulation, the bottom well was filled with 500 μL DMEM with 10% FBS. After incubation, nonmigrating cells were removed and membranes were stained with crystal violet. Photomicrographs of 3 regions were captured from duplicated inserts, and the numbers of cells were counted and normalized to the control group. All experiments were repeated 3 times.

Animals and tumor cell injection

Male athymic BALB/c nude mice were purchased from National Laboratory Animal Center, and Bio LASCO Taiwan Co. Mice were injected with 1 × 10⁶ PC3 cells stably expressing Vav3 or control shRNA in the prostate as described (see Supplementary Material; ref. 26). Two runs of implantation were carried out independently and 6 to 8 mice were used per group each time. Results from the experiments were combined as the final data. Mice were sacrificed 28 to 35 days after tumor cell injection. Tissue specimens were fixed, paraffin-embedded, serially sectioned, and stained with hematoxylin and eosin (H&E). In the second run of mouse injections, bone marrow cells from mouse thigh bones were collected and cultured in DMEM with 10% FBS to detect bone metastatic cells from PC3 tumor cells in the prostate.

Immunohistochemistry

Paraffin-embedded tissue sections of human prostate cancer specimens were obtained from Chi-Mei Medical Center (Tainan, Taiwan; using an Institutional Review Board–approved protocol) or from commercial prostate cancer tissue arrays (US Biomax, Inc. and SuperBioChips). The slides were stained with anti-Vav3 or anti-EphA2 using an automatic slide stainer BenchMark XT (Ventana Medical Systems).
Patients and follow-up

Fifty radical prostatectomy specimens, no higher than stage IIB, were retrospectively identified from Chi-Mei Medical Center between 1998 and 2005. The detailed information on these specimens is provided in the Supplementary Material.

Results

Upregulation of Vav3 and correlation with increased migration/invasion in selected androgen-independent prostate cancer cells

To evaluate Vav3 expression and its possible relation to migration/invasion, the present study tested several prostate cancer cell lines. Real time RT-PCR and Western blot analysis both revealed higher Vav3 mRNA and protein expression in the androgen-independent lines, PC3 and C4-2 (27), than in the androgen-dependent line, LNCaP (Fig. 1A and B). These data are consistent with previous reports of Vav3 overexpression in prostate cancer (18, 19). Similarly, upregulation of EphA2 occurred in the 2 androgen-independent lines, especially in PC3 (Fig. 1A and B).

Subsequent analyses evaluated the possible association of migration/invasion with Vav3 expression, observing that both migration and invasion occurred to significantly greater extents in the high Vav3-expressing cells (C4-2 and PC3) than in the low Vav3-expressing LNCaP cells (Fig. 1C and D). These results suggest that Vav3 upregulation might lead to enhanced migration and invasion abilities.

Role of Vav3 in promotion of migration/invasion by way of Rac1 activation in LNCaP cells

To explore the role of Vav3 in migration/invasion, we first showed that overexpression of Vav3 or Vav3-(6-10), the constitutively activated mutant (Fig. 2A; ref. 3), increased migration/invasion (Supplementary Fig. S1A), which correlated with Rac1 activation (Supplementary Fig. S1B) in NIH3T3 cells. In prostate cancer cells, individual transfection of low Vav3-expressing LNCaP cells (Fig. 1B) with Vav3 or Vav3-(6-10) (Fig. 2B) resulted in significantly increased migration/invasion (Fig. 2C and D). The ability to induce both migration and invasion was much higher for Vav3-(6-10) than for full-length Vav3, indicating that the stimulus for Vav3 activation might be important in Vav3-, but not Vav3-(6-10), mediated migration/invasion.

Subsequent testing of the small Rho GTPases revealed that the dominant-negative form of Rac1 (dnRac1) significantly inhibited Vav3-(6-10)-induced migration/invasion, whereas dnCdc42 and dnRhoA did not (Figs. 2E and F). Using siRNAs specifically targeting these Rho GTPases excluded the non-selective effects caused by the dominant-negative form (Supplementary Fig. S2A). Consistently, only cells with reduced Rac1 expression displayed significant inhibition of Vav3-(6-10)-induced migration/invasion (Fig. S2B and S2C). Consistent with increased migration/invasion, LNCaP cells expressing Vav3, and especially Vav3-(6-10), displayed increased GTP-Rac1, the active form of Rac1 (Fig. 2G). Overall, these results indicate that Vav3 promotes prostate cancer migration/invasion, mainly through activation of Rac1.

The ephrinA1 ligand stimulation of EphA2 receptor results in recruitment of Vav3, tyrosine phosphorylation of Vav3, Rac1 activation, and increased migration/invasion

Activation of Vav3 occurs by way of Y173 phosphorylation by several RTKs, including insulin, insulin-like growth factor (IGF) 1, EGF receptors, Ros (3), and EphA2 (17). To identify which RTK(s) functions as the upstream activator for Vav3 in prostate cancer cells, the present study tested several RTKs. The EGF

Figure 1. Overexpression of Vav3 and correlation with migration/invasion in androgen-independent prostate cancer cells. A, real-time RT-PCR analysis of mRNA levels in LNCaP, C4-2, and PC-3 cells. Data are presented as normalized mean ± SD (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001. B, Western blot analysis of Vav3 and EphA2 protein levels in LNCaP, C4-2, and PC3 cells. Histograms represent normalized mean ± SE (n = 3). Cell migration (C) and invasion (D) assays were carried out. Cells were incubated for 24 hours for migration and 48 hours for invasion assays. DMEM with 10% FBS served as a chemoattractant. Numbers represent normalized mean ± SD (n = 8). *P < 0.05; **P < 0.01; ***P < 0.001.
receptor (EGFR) or Met (hepatocyte growth factor receptor) activation, but not the insulin receptor (IR), is able to stimulate Vav3 activity in LNCaP or PC3 cells to varying extents (Supplementary Fig. S3B–S3D). Activation of Vav3 by these receptors might contribute to Vav3-mediated cell migration/invasion.

Other than those RTKs, the expression of EphA2 correlated with Vav3 in prostate cancer cells (Fig. 1A and B). It stimulated endogenous Vav3 tyrosine phosphorylation in the presence of the preclustered EphA2 ligand, ephrinA1-Fc, in PC3 cells (Fig. 3A), and so was the overexpressed Vav3 in both PC3 and LNCaP cells (Supplementary Fig. S3A). Endogenous Vav3 was associated with EphA2 upon ephrinA1-Fc stimulation (Fig. 3B). This confirmed the ability of activated EphA2 to bind Vav2/3 (28). Transfection of LNCaP cells with EphA2 or EphA2 plus Vav3 further tested whether EphA2 activation regulates Vav3-Rac1 signaling. By ephrinA1 stimulation, Rac1 was activated in EphA2-expressing cells; active Rac1 was markedly increased in cells expressing EphA2 together with Vav3 (Fig. 3C). Consistently, upon ephrinA1-Fc stimulation, migration/invasion was significantly greater in LNCaP cells overexpressing Vav3 than in unstimulated cells (Fig. 3D).

Reducing EphA2 expression in PC3 cells tested the requirement for EphA2 in Vav3 activation (Fig. 3E) with results showing markedly reduced ephrinA1-induced Vav3 tyrosine phosphorylation (Fig. 3F). The slightly decreased Vav3 upon siEphA2 has been reproducibly observed. The reason for this is unclear. However, the decrease of phosphorylated Vav3 is much greater than that of Vav3. These data indicate that EphA2 serves as a key upstream activator for Vav3-Rac1 signaling, leading to enhanced migration/invasion in prostate cancer cells.
Knockdown of Vav3 in PC3 cells results in decreased migration/invasion and attenuated Rac1 activity

Knockdown experiments on high Vav3-expressing PC3 cells (Fig. 1A and B) evaluated the effects of reducing Vav3 expression on migration/invasion. The PC3 cells with reduced Vav3 protein expression (Fig. 4A) showed more than 50% reduction in serum-stimulated migration/invasion (Fig. 4B) and concordantly reduced GTP-Rac1 levels (Fig. 4C). Expression of several matrix metalloproteinases (MMP) decreased in PC3 cells upon Vav3 knockdown (Supplementary Fig. S4A), indicating the potential role of Vav3 in the regulation of MMP expression. Knockdown of Vav3 resulted in attenuated EphrinA1-induced Rac1 activity (Supplementary Fig. S4B) and decreased migration/invasion (Supplementary Fig. S4C and S4D). Results indicated that the activation of Rac1 by EphA2 signaling was less significant than serum-induced activation, suggesting that other RTKs in serum may also mediate Vav3-Rac1 signaling in PC3 cells, such as EGFR or Met (Supplementary Fig. S3B and S3D).

The C4-2 cells which expressed endogenous Vav3 plus AR (Fig. 1A and B) showed attenuated EphA2-mediated Rac1 activity (Supplementary Fig. S4F) and decreased migration/invasion (Supplementary Fig. S4G and S4H) with Vav3 knockdown (Supplementary Fig. S4E). Overall, results indicate that Vav3 plays an important role in migration/invasion in prostate cancer cells.

Prostate orthotopic implantation of PC3 cells with reduced Vav3 expression slowed primary tumor growth and reduced incidence of para-aortic lymph nodes and bone metastases

To further investigate the role of Vav3 during prostate cancer metastasis in vivo, approaches of xenografted orthotopic implantation in nude mice were established. Nude mice were orthotopically injected with control or Vav3 knockdown...
OF6

Cancer Res; 72(12) June 15, 2012

Lin et al.

PC3 cells into the prostate. Mice were sacrificed 28 to 35 days after injection. Table 1 summarizes the results of tumor growth and metastasis. In the shVav3 group, the tumor volumes reduced to 50% of the volumes in the shControl group. However, the in vitro cell proliferation rates in shControl and shVav3 cells were similar (Supplementary Fig. S5C). As expected, Vav3 expression was lower in the shVav3 group than in the shControl group (Fig. 5A and D). Figure 5B shows the histological morphology of the primary tumors.

Previous research has shown well-characterized PC3 cells as the metastatic model for prostate orthotopic implantation (26), observing the development of local invasion, such as seminal vesicle and lymph node metastases, 40 days after injection (26), observing the development of local invasion, such as the metastatic model for prostate orthotopic implantation (26). Figure 5A shows the expected, Vav3 expression was lower in the shVav3 group than in the shControl group (Fig. 5A and D). Figure 5B shows the histological morphology of the primary tumors.

Further analyses verified Vav3 levels in the primary and metastatic tumors of mice injected with PC3-shControl or PC3-shVav3 cells. All mice in the PC3-shControl group developed para-aortic lymph node metastases (8 of 8); 6 of 15 mice did so in the PC3-shVav3 group. Real time RT-PCR results indicated a 3-fold reduction in Vav3 expression in primary tumors in the shVav3 group compared with the control group (Fig. 5D). Two of the metastasized lymph nodes in the shVav3 group displayed increased Vav3 expression compared with their respective primary tumors; the others displayed low Vav3 similar to their primary tumors. These data confirm knockdown of Vav3 expression in the PC3-shVav3 tumors

Table 1. Tumorigenicity and incidence of prostate cancer metastases by PC3 cell implantation in the prostate in nude mice

<table>
<thead>
<tr>
<th>Xenograft</th>
<th>PC3-shControl</th>
<th>PC3-shVav3</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average mouse weight, g</td>
<td>22.31 ± 1.56</td>
<td>21.49 ± 2.06</td>
<td>0.27</td>
</tr>
<tr>
<td>Average tumor size, mm³</td>
<td>88.95 ± 55.79</td>
<td>46.95 ± 31.25</td>
<td>0.03</td>
</tr>
<tr>
<td>Average para-aortic lymph nodes size, mm³</td>
<td>5.32 ± 3.03</td>
<td>0.73 ± 0.48</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Para-aortic lymph nodes metastases (%)</td>
<td>100 (12/12)</td>
<td>35.7 (5/14)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Microscopic bone metastases (%)</td>
<td>50 (3/6)</td>
<td>0 (0/8)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Figure 4. Reduced Vav3 expression in PC3 cells decreases Rac1 activation and cell migration/invasion. A, Western blot analysis showed Vav3 expression in PC3 cells with control or Vav3 siRNA transfection. Histograms represent normalized mean ± SD (n = 3). B, the PC3 cells transfected with control or Vav3 siRNA were incubated for 16 hours for migration and 24 hours for invasion assays. DMEM with 10% FBS served as a chemoattractant. Numbers represent normalized mean ± SD (n = 3). C, Western blot analysis of GST-PAK immunoprecipitates (active GTP-Rac1) or lysates in PC3 cells with reduced Vav3 expression. The small Rho GTPase, caRac1, was used as a positive control. Histograms represent normalized mean ± SE (n = 3). wt, wild-type.
try, with grouping using the tumor cancer. Vav3 and EphA2 in the development of advanced prostate expression. These data suggest the possible involvement of metastatic (80%) samples displayed both Vav3 and EphA2 in Fig. 6B, a high percentage of stage IV (85.7%) and well with the prostate cancer stage (Fig. 6A). As shown tumors. The staining intensities of both proteins correlated stage tumors, 15 metastatic tumors, and 16 adjacent non-

The present study indicates the significance of RTK-mediated activation of Vav3, especially the EphA2-Vav3-Rac1 signaling axis, in prostate cancer metastasis. Upon activation of EphA2 or other RTKs, Vav3 is recruited to the receptor(s) and is tyrosine-phosphorylated. The activated Vav3 then promotes

discussion

The present study indicates the significance of RTK-mediated activation of Vav3, especially the EphA2-Vav3-Rac1 signaling axis, in prostate cancer metastasis. Upon activation of EphA2 or other RTKs, Vav3 is recruited to the receptor(s) and is tyrosine-phosphorylated. The activated Vav3 then promotes

Expression of Vav3 and EphA2 correlates with prostate cancer progression

In previous studies, both Vav3 and EphA2 were upregulated in prostate cancer specimens (13, 18). However, these studies did not describe the relationship of this upregulation to different tumor stages. The present study evaluated Vav3 and EphA2 expression in prostate cancer specimens with defined stages and grades using immunohistochemistry, with grouping using the tumor–node–metastasis (TNM) staging system. The evaluations included 75 primary prostate tumors, 15 metastatic tumors, and 16 adjacent non-tumors. The staining intensities of both proteins correlated well with the prostate cancer stage (Fig. 6A). As shown in Fig. 6B, a high percentage of stage IV (85.7%) and metastatic (80%) samples displayed both Vav3 and EphA2 expression. These data suggest the possible involvement of Vav3 and EphA2 in the development of advanced prostate cancer.

The potential use of Vav3 as a prognostic marker of early-stage prostate cancer

Analysis of Vav3 expression in 50 primary radical prostatectomy specimens, of stage no higher than IIB, evaluated the prognostic potential of Vav3 expression in early prostate cancer. The overall 7-year biochemical failure-free survival rate was significantly lower in patients with high Vav3 expression than in those with low Vav3 expression (60% vs. 90%; Fig. 6C). In patients with prostate cancer, the return of increased levels of prostate-specific antigen (PSA) indicates treatment failure, providing clinical evidence of tumor recurrence (29). The Vav3 oncoprotein might, therefore, provide a suitable clinical prognostic marker for the early prediction of prostate cancer recurrence following radical prostatectomy.
Rac1 activation among others, such as increased MMPs, to regulate migration/invasion of prostate cancer cells in the metastatic processes (Supplementary Fig. S6).

The Vav3 oncoprotein is a signaling transducer downstream of RTKs in various signaling pathways. The study findings indicated that EphA2 is a key upstream RTK activator of Vav3 in prostate cancer cells (Fig. 3A–D). In a similar manner to Vav3, EphA2 expression markedly increased in androgen-independent prostate cancer cells (Fig. 1B) and in advanced-stage human prostate cancers (Fig. 6A and B). In a breast cancer model, EphA2-deficient mice displayed decreased tumor volume, microvasculature density, and lung metastasis (30). The EphA2 receptor might, therefore, play a significant role in prostate cancer metastasis by activating Vav3-Rac1 signaling. Other RTKs might also mediate prostate cancer progression through Vav3 signaling, such as EGF and Met receptors (Supplementary Fig. S3B and S3D).

The EphA2 receptor is an important oncoprotein in prostate cancer progression (16). However, the mechanism by which EphA2 promotes prostate cancer progression remains unclear. Previous research has suggested that EphA2 overexpression promotes PC3 cell motility in a ligand-independent manner (31), whereas ephrinA1-induced kinase activation leads to inhibition of serum-induced AKT phosphorylation (31) and integrin function (32), resulting in decreased migration and adhesion ability. In the study of Pratt and Kinch, however, ephrinA1-induced activation of EphA2 stimulated mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase signaling (33). Cancer cells with the EphA2 kinase–deficient mutant have shown reduced metastasis in prostate carcinoma (16) and breast cancer (10), indicating the importance of EphA2 kinase–dependent signaling in metastasis. The results of the present study indicate that Vav3 is activated in an EphA2 kinase–dependent manner (Fig. 3A

**Figure 6.** Correlation of EphA2 and Vav3 expression with late-stage prostate cancer and patients with higher Vav3 expression show higher probability of prostate cancer posttreatment recurrence. A and B, immunohistochemical (IHC) analysis of Vav3 and EphA2 expression in different TNM stages of human prostate cancer (B). Representative microphotographs of immunohistochemical staining (×200; A). The staining intensities were evaluated by 2 independent investigators. N, adjacent non-tumor; M, distant metastasis. C, retrospective analysis of Kaplan–Meier biochemical failure-free survival curves from 50 patients with cancer stages no higher than IIB. Patients were grouped into low Vav3-expressing versus high Vav3-expressing groups for follow-up analysis.

**Table 1.** Summary of IHC analysis of Vav3 plus EphA2 expression in primary prostate tumor specimens based on stage

<table>
<thead>
<tr>
<th>Stage</th>
<th>Vav3-positive (%)</th>
<th>P</th>
<th>EphA2-positive (%)</th>
<th>P</th>
<th>Both-positive (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>18.8 (3/16)</td>
<td>--</td>
<td>51.2 (5/16)</td>
<td>--</td>
<td>18.8 (3/16)</td>
<td>--</td>
</tr>
<tr>
<td>II</td>
<td>26.7 (4/15)</td>
<td>0.612</td>
<td>80.0 (12/15)</td>
<td>0.005</td>
<td>26.7 (4/15)</td>
<td>0.612</td>
</tr>
<tr>
<td>III</td>
<td>43.4 (13/30)</td>
<td>0.128</td>
<td>52.8 (27/53)</td>
<td>0.103</td>
<td>34.7 (18/52)</td>
<td>0.236</td>
</tr>
<tr>
<td>IV</td>
<td>85.7 (6/7)</td>
<td>0.008</td>
<td>85.7 (6/7)</td>
<td>0.615</td>
<td>85.7 (6/7)</td>
<td>0.008</td>
</tr>
<tr>
<td>M</td>
<td>93.3 (14/15)</td>
<td>&lt; 0.001</td>
<td>86.7 (13/15)</td>
<td>0.001</td>
<td>80.0 (12/15)</td>
<td>0.003</td>
</tr>
</tbody>
</table>
and B), resulting in increased cell migration/invasion in LNCaP (Fig. 3D), C4-2 (Supplementary Fig. S4G and S4H), and PC3 (Supplementary Fig. S4C and S4D) cells under serum-free conditions. Although the exact mechanism by which EphA2 promotes cancer progression in vivo remains unclear, these data suggest the significant involvement of EphA2 kinase–dependent signaling in prostate cancer metastasis.

The Rac1 protein stimulates the formation of lamellipodia and membrane ruffles to control cell motility (34). Accumulating evidence indicates the important role of Rac1-mediated signaling in malignant transformation (35, 36). Elevated expression of Rac1 in prostate cancer (37) and its suppression of the cyclin-dependent kinase inhibitor p21 (CIP1) in prostate cancer cells (38) suggest the involvement of Rac1 in prostate cancer progression. Several Rac-specific GEFs have been linked to tumor progression, such as Tiam1 (39) and DOCK3 (40). In addition, Gao and colleagues have developed a small inhibitor, which specifically interferes with interaction between Rac1 and its GEFs (41). Results from the present study indicate the significance of another Rac-specific GEFs, Vav3, in prostate cancer cell migration/invasion (Figs. 2–4) through the Vav3–Rac1 signaling axis. Apart from effects on cell motility, preliminary data suggest that the increased invasive ability might derive from the regulation of production of MMPs (Supplementary Fig. S4A); and in previous studies, Rac1 has been shown to induce expressions of MMPs (42, 43). Overall, these data provide evidence to suggest that a mechanism in prostate cancer metastasis is Rac1-mediated.

In the study of Liu and colleagues, which developed prostate-specific Vav3 transgenic mice, some of the adult mice developed tumors (24) with no distant metastases. Cancer progression involves multiple mutation events; thus, it is not surprising that Vav3 is not the only driving force in the metastatic process. In the present study, Vav3 knockdown in PC3 cells, which have intrinsic metastatic potential, led to significantly lower incidence of para-aortic lymph node and bone metastasis (Table 1), the prevalent sites of prostate cancer metastasis.

In the orthotopically implanted mice, the primary tumors in the PC3-shVav3 group were 2 times smaller than those in the shControl group. However, the growth rates of PC3-shControl and PC3-shVav3 cells were similar (Supplementary Fig. SSC).

The cause of the differences in tumor volume between these 2 groups is unclear. One possibility is angiogenesis. Hunter and colleagues have shown EphA2-Vav2/3-Rac1 signaling involvement in angiogenesis (17), suggesting a role for Vav3 in angiogenesis. The PC3-shVav3 tumor might be deficient in angiogenesis processes because of reduced Vav3 expression. Another possibility is that the in vivo tumor microenvironment favors the growth of tumor cells in the presence of Vav3 signaling; for example, by way of Vav3-mediated cell–matrix interaction. The observations of the present study of increased Vav3 expression in some of the lymph node metastases from the shControl and shVav3 groups (Fig. 5D) support the role of Vav3 in promoting prostate cancer metastasis.

Biochemical failure-free survival is a valuable clinical parameter for the risk of development of local recurrence, distant metastasis, and prostate cancer–specific mortality in prostate cancer (29). In the present study, approximately 40% of patients with high Vav3 expression developed PSA recurrence within 80 months of radical prostatectomy (Fig. 6C), whereas the low Vav3 group had almost no recurrence of elevated PSA levels. These patients’ cancer stages were no higher than stage IIB, indicating the potential use of Vav3 prognostic marker of early disease outcome following initial treatment. Given the important clinical implications of this observation, further analysis including a greater number samples is warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank the Pathology Core Laboratory of the National Health Research Institutes for H&E and immunohistochemical staining and Dr. Simon Hall (Mount Sinai School of Medicine) for the gift of C4-2.

Grant Support

This work was supported by grants from the National Health Research Institutes, Taiwan (99A1-MQPP11-014) and the Department of Health, Taiwan (DOH100-TD-111-004).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 28, 2011; revised February 20, 2012; accepted March 5, 2012; published OnlineFirst June 1, 2012.

References


Vav3-Rac1 Signaling Regulates Prostate Cancer Metastasis with Elevated Vav3 Expression Correlating with Prostate Cancer Progression and Posttreatment Recurrence

Kai-Ti Lin, Jianli Gong, Chien-Feng Li, et al.

Cancer Res  Published OnlineFirst June 1, 2012.

Updated version  Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-11-2502

Supplementary Material  Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2012/06/13/0008-5472.CAN-11-2502.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.