Compensatory Upregulation of Tyrosine Kinase Etk/BMX in Response to Androgen Deprivation Promotes Castration-Resistant Growth of Prostate Cancer Cells

Bojie Dai\(^1\), Hege Chen\(^1\), Shengjie Guo\(^4\), Xi Yang\(^1\), Douglas E. Linn\(^1\), Feng Sun\(^1\), Wei Li\(^1\), Zhiyong Guo\(^1\), Kexin Xu\(^1\), Oekyung Kim\(^1\), Xiangtian Kong\(^3\), Jonathan Melamed\(^3\), Shaopeng Qiu\(^5\), Hegang Chen\(^2\), and Yun Qiu\(^1\)

Abstract

We previously showed that targeted expression of non–receptor tyrosine kinase Etk/BMX in mouse prostate induces prostate intraepithelial neoplasia, implying a possible causal role of Etk in prostate cancer development and progression. Here, we report that Etk is upregulated in both human and mouse prostates in response to androgen ablation. Etk expression seems to be differentially regulated by androgen and interleukin 6 (IL-6), which is possibly mediated by the androgen receptor (AR) in prostate cancer cells. Our immunohistochemical analysis of tissue microarrays containing 112 human prostate tumor samples revealed that Etk expression is elevated in hormone-resistant prostate cancer and positively correlated with tyrosine phosphorylation of AR (Pearson correlation coefficient \(\rho = 0.71, P < 0.0001\)). AR tyrosine phosphorylation is increased in Etk-overexpressing cells, suggesting that Etk may be another tyrosine kinase, in addition to Src and Ack-1, which can phosphorylate AR. We also showed that Etk can directly interact with AR through its Src homology 2 domain, and such interaction may prevent the association of AR with Mdm2, leading to stabilization of AR under androgen-depleted conditions. Overexpression of Etk in androgen-sensitive LNCaP cells promotes tumor growth while knocking down Etk expression in hormone-insensitive prostate cancer cells by a specific shRNA that inhibits tumor growth under androgen-depleted conditions. Taken together, our data suggest that Etk may be a component of the adaptive compensatory mechanism activated by androgen ablation in prostate and may play a role in hormone resistance, at least in part, through direct modulation of the AR signaling pathway. Cancer Res; 70(13); 5587–96. ©2010 AACR.

Introduction

Hormonal therapy has been the treatment of choice for patients with advanced metastatic prostate cancer since Charles Huggins and his colleagues first demonstrated the effectiveness of androgen ablation therapy in 1941. However, a majority of patients will inevitably develop recurrent hormone-resistant tumors, rendering the conventional therapy ineffective. Therefore, understanding the molecular basis underlying progression to a castration-resistant state is the first step toward developing therapies for this lethal form of prostate cancer (1).

Progression to castration resistance is a multifactorial process by which prostate cancer cells acquire the ability to survive and proliferate under low levels of androgenic stimuli. The androgen receptor (AR) is primarily responsible for mediating the physiologic effects of androgens by binding to specific DNA sequences, known as androgen response elements (ARE), which regulate transcription of androgen-responsive genes (2). Studies have shown that AR continues to be expressed and AR signaling remains intact in castration-resistant tumors (3). Potential mechanisms by which AR may be reactivated in the androgen-deprived environment include mutations and amplification of the AR gene, increased expression of steroid metabolism enzymes, steroid hormone receptor coactivators, and elevated levels of growth factors and cytokines (4). Increased AR expression was shown to be associated with the development of anti-androgen therapy resistance (5). Recently, we found that phosphorylation of AR Y534 induced by Src kinase may contribute to androgen-independent activation of AR or sensitize AR to low levels of hormone (6). Our immunohistochemical survey showed a moderate correlation of AR Y534 phosphorylation with Src kinase activity in human prostate tumor tissues, suggesting that Src is one of the tyrosine kinases that phosphorylates AR. Likely, additional kinases may also be involved in regulation of AR activity. This possibility was supported by an
independent study on the Ack-1 kinase, which has been shown to induce AR tyrosine phosphorylation (7). A recent report showed that phosphorylation of Y534 leads to stabilization of AR induced by a neuroendocrine-derived parathyroid hormone–related protein (8).

Epithelial and endothelial tyrosine kinase (Etk, also known as BMX) is a non–receptor tyrosine kinase and a downstream effector of Src and phosphoinositide 3-kinase (PI3K; refs. 9, 10). Etk/BMX contains an NH2-terminal pleckstrin homology domain, Src homology 3 (SH3) domain, SH2 domain, and COOH-terminal tyrosine kinase domain. Etk has been implicated in various biological processes, including proliferation, differentiation, apoptosis, and cell migration. Etk/BMX expression elevated in several aggressive metastatic carcinoma cell lines suggests that Etk/BMX may be involved in the development and progression of prostate cancer (11). The interaction between Etk and FAK is involved in integrin signaling and may play a role in tumor metastasis of prostate cancer cells (11, 12). Etk/BMX is upregulated during stress (e.g., radiation, wound healing) in endothelial cells and skin keratinocytes (13, 14), and mediates regulation of expression of stress-induced adaptive genes such as VEGF, PAI-1, and iNOS through multiple signaling cascades in different cell systems (15). Etk is activated by interleukin 6 (IL-6) in prostate cancer cells through the PI3K pathway and has been implicated in neuroendocrine differentiation (9). The synergism between Etk and Pim-1 seemed to be involved in IL-6–induced ligand-independent activation of AR in prostate cancer cells (16). We also showed that Etk directly interacts with the tumor suppressor p53, and such interaction results in a bidirectional inhibition of the functions of both proteins (17). Furthermore, Etk is required for growth of prostate cancer cells induced by neuropeptides such as bombesin and neurotensin (18). However, the functional significance of Etk overexpression in castration-resistant prostate cancer remains unknown.

In this report, we show that Etk expression is elevated in response to androgen ablation in both human and mouse prostates. Our study suggests that Etk may be a component of the adaptive compensatory mechanism activated by androgen ablation in the prostate and may play a role in the development of hormone-resistant prostate cancer, at least in part, by directly modulating AR function.

Materials and Methods

Tissue microarray, immunohistochemical analysis, and statistical analysis

Two intermediate-density prostate tissue arrays were prepared by the NYU Cooperative Prostate Cancer Tissue Resource and consisted of a total 112 cases (four cores per case), including 18 hormone-resistant and 18 hormone-naïve transurethral resection (TURP) specimens of prostate from patients with clinically advanced prostate cancer, and 76 cases of hormone-naïve prostate cancer tissue (Gleason sum 6–10) from the radical prostatectomy specimens of patients with clinically localized prostate cancer. Hormone-naïve and hormone-resistant states were determined as follows: (a) Patients who had earlier undergone surgical orchiectomy or medical hormone-suppressive therapy at least 6 months before the procedure were considered as hormone resistant; (b) patients who did not receive hormonal therapy before TURP were considered as hormone naïve. Tissue specimens were from the archival paraffin block inventory of the NYU Cooperative Prostate Cancer Tissue Resource. All cases upon collection into the resource (under an institutional review board–approved protocol) had repeat pathology characterization of tissues and review of medical records. The Vectastain Elite ABC Kit (Vector Laboratories) was used for immunohistochemical staining according to the protocol recommended by the manufacturer. Finally, sections were counterstained with hematoxylin, dehydrated, and mounted. Immunohistochemical staining was assessed independently by two pathologists (X.K. and J.M.), and a consensus of grading was reached. Immunostaining was graded using a two-score system based on intensity score and proportion score as described previously (6). The immunoreactive score for each case was quantified by the average of four cores. Statistical analyses were carried out by using the SAS version 9.0 software (SAS Institute).

Animal dissection, histologic analysis, immunoprecipitation, and Western blot

Prostates were dissected under a dissecting microscope as described previously (19). Immunohistochemistry analysis was carried out with the following antibodies diluted in PBS: anti-Etk, anti-AR, and anti-pARY534 (6). Negative controls were included in each assay. Prostate tissues were extracted by using T-PER protein extraction reagent (Pierce). Antibodies were added to lysates and incubated for 1 to 3 hours at 4°C. Immunoblotting was performed as described previously (12). Anti-Etk was used at 1:20,000, whereas anti-Erk1/2, anti-actin (C2), and anti-AR (N-20; Santa Cruz Biotechnology) were used at 1:2,000. Anti–phospho SrcY416 (anti-pSrc; Cell Signaling) and anti-FLAG antibody M2 (Sigma) were used at 1:4,000. The phosphospecific antibody for ARY534 (anti-pARY534), described previously (6), was used at 1:2,000.

Quantitative real-time reverse transcription-PCR

Quantitative real time reverse transcription-PCR (RT-PCR) was performed as described previously (6). The primer sequences used for PSA were TCTGCGCGGTGTTCGTG and GCCGACCCAGCAAGATCA; AR, CCTGGGCTTCTC-GCAACTTACAC and GGACTTGTGCATGCGGTACTCA; Etk, CTCTAAATGTGTGTGGAG and TGGAGCTGAC-CACTTGAAT; 18S, TTGACGGAAAGGCCACCCAGC and GCACCACCAACCGATAT; actin, GCTATCCAGG-CCTGGTACTAC and TGGTCAGGACAG TCC; the relative abundance of each target transcript was quantified by using the comparative \( \Delta \Delta Ct \) with actin as an internal control (6).

Chromatin immunoprecipitation

LNCaP cells were cultured in phenol red–free RPMI 1640 containing 5% charcoal-stripped serum for 3 days and then serum-starved for 16 hours, followed by treatment with...
vehicle control (ethanol), 10 nmol/L dihydrotestosterone (DHT), or 20 ng/mL IL-6 for 1 hour. Chromatin immunoprecipitation (ChIP) was performed by using anti-AR or IgG as described previously (6, 20).

The PCR primers were as follows: ARE-P, 5′-agcctagtctgtaggatgtaaga-3′ and 5′-tctctgtaaatggattgtc-3′; ARE1, 5′-aggctctgagaacgcatgttct-3′ and 5′-gcgtatcctgctgtaaa-taacct-3′; ARE2, 5′-attaaccctgtccctctggta-3′ and 5′-ataggctct tcgtgtaaaaggg-3′; ARE3, 5′-tagggtaa agggctgac-3′ and 5′-aagagcaatggtgacgtagg-3′; ARE4, 5′-tctctcttatcaactgta-3′ and 5′-agttttttccaatgtcactgg-3′.

**Cell culture and in vivo tumor growth in the xenograft models**

LNCaP and COS-1 cells were purchased from the American Type Culture Collection. Hormone-resistant prostate cancer cell lines C-81 and CWR-R1 were kindly provided by Drs. Ming-Fong Lin (ref. 21; Department of Biochemistry and Molecular Biology, Nebraska Medical Center, Omaha, NE), Christopher Gregory, and Elizabeth Wilson (ref. 22; both at Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC), respectively. LNCaP and C-81 cells were maintained in RPMI 1640 with 10% fetal bovine serum (FBS). COS-1 cells were grown in DMEM medium with 10% FBS. LAPC-4 cells (kindly provided by Dr. Charles Sawyer; Human Oncology and Pathogenesis Program, Memorial Sloan-Kettering Hospital, New York, NY) were maintained in IMEM supplemented with 15% FBS. CWR-R1 cells were maintained in RPMI 1640 with 10% heat-inactive FBS. Transfections were carried out using FuGENE 6 (Roche) or Lipofectamine 2000 (Invitrogen) following the manufacturer’s instruction. LNCaP cells overexpressing T7-Etk were described previously (17). Lentivirus infections were carried out as described previously (17). Tumor growth in severe combined immunodeficient (SCID)/nude mice was carried out by s.c. inoculation of 10⁶ prostate cancer cells mixed with Matrigel as described previously (6). All procedures involving animals were approved by Institutional Animal Care and Use Committee of the University of Maryland.

**Results**

**Etk expression is increased in prostatic epithelium in response to androgen ablation**

In an effort to examine the changes in mouse prostate in response to castration, we observed that the Etk expression level was dramatically increased along with IL-6, which is up-regulated after castration (Fig. 1A, left). Immunohistochemical analysis revealed an increase of Etk staining in both epithelial and stromal compartments in prostates of castrated mice. We also performed real-time RT-PCR analysis and detected a significant and progressive increase of Etk mRNA in the castrated mouse prostate (Fig. 1A, right). Increased Etk expression in prostate seemed to be reversible when the castrated animals were treated with androgen replacement (Fig. 1B). Etk expression was also increased in xenograft tumors derived from both LNCaP and LAPC4 cells after castration (Fig. 1C). Furthermore, DHT treatment reduced the level of Etk mRNA in LNCaP cells in a concentration-dependent manner whereas an androgen antagonist, bicalutamide, had an opposing effect (Supplementary Fig. S1). These data suggest that Etk expression is negatively regulated by androgens. Interestingly, multiple putative ARE sites were found in the promoter region and introns of human ETK/BMX gene (Fig. 1C). ChIP assays revealed that both DHT and IL-6 could recruit AR to the ARE sites located in the promoter region and the ARE4 site in intron 2, whereas only DHT was able to induce AR binding to the ARE2 and ARE3 sites located in intron 1. This suggests a differential recruitment of AR to these ARE sites in ETK locus in response to DHT or IL-6 (Fig. 1C). We also observed varied effects on occupancy of RNA polymerase II and coactivator SRC1 in this region in cells treated with DHT or IL-6 (Supplementary Fig. S2). This may provide mechanistic insight into differential regulation of Etk expression by androgen and IL-6.

**Etk expression is elevated in hormone-resistant prostate tumors and correlates with AR Y534 phosphorylation**

To examine Etk expression in human prostate tumors, we performed immunohistochemistry analysis on human prostate tissue arrays containing 94 hormone-naïve and 18 hormone-resistant samples. As summarized in Fig. 2A, Etk immunoreactivity is dramatically increased in both the cytoplasm and nucleus of human hormone-resistant prostate tumor samples (mean score, 4.30 ± 0.45 and 5.42 ± 0.33, respectively) compared with hormone-naive samples (mean score, 0.57 ± 0.15 and 2.44 ± 0.22, respectively). In a previous study, we showed that phosphorylation of AR Y534 is elevated in hormone-resistant prostate tumors and that there is a moderate correlation between AR Y534 phosphorylation and active Src kinase. Src and Etk share some common substrates due to the high degree of homology of their kinase domains; thus, we examined whether increased Etk expression could also contribute to AR Y534 phosphorylation. Interestingly, correlation analysis revealed that Etk expression level in human prostate tumors is strongly correlated with phosphorylation of AR Y534 (Pearson correlation coefficient ρ = 0.71, P < 0.0001). Figure 2B shows representative fields of human prostate tissue arrays. We then tested whether Etk can induce AR Y534 phosphorylation in cotransfection experiments using active SrcY527F mutant as a positive control. As shown in Fig. 2C, wild-type Etk (EtkWT), but not kinase-inactive Etk (EtkKQ), induced AR tyrosine phosphorylation, although the magnitude of AR phosphorylation induced by Etk was not as great as that induced by active Src. We also found that phosphorylation of AR Y534 was increased in Etk transgenic prostates compared with wild-type (WT) control prostates, whereas Src activity in Etk transgenic prostates seemed to remain largely unchanged (Fig. 2D). On the other hand, knockdown of endogenous Etk expression by a specific shRNA diminished AR Y534 phosphorylation in CWR-R1 cells (Supplementary Fig. S3). Taken together, these data suggest that Etk may be another kinase, in addition to Src, responsible for phosphorylating AR Y534 in prostate cells.
Castration promotes the association between Etk and AR in a phosphorylation-dependent manner

To assess whether Etk could play a role in regulation of AR signaling at the castratory levels of testosterone, we examined the association between Etk and AR in hormone-sensitive and hormone-resistant tumor xenografts in coimmunoprecipitation experiments. As shown in Fig. 3A, the association between Etk and AR was increased in hormone-resistant tumor xenografts compared with their hormone-sensitive counterparts. To further investigate which domain(s) of Etk are involved in its interaction with AR, we performed glutathione S-transferase (GST) pull-down experiments. Surprisingly, it seemed that only the fusion proteins containing the SH2 domain of Etk (GST-EtkSH2 and GST-EtkΔKin) were able to interact with AR (Fig. 3B), implying that AR tyrosine phosphorylation may be involved in this interaction. To investigate whether AR tyrosine residues contribute to the association between Etk and AR, we examined several AR phosphorylation mutants that were established in our previous study on Src kinase-induced AR phosphorylation in vivo (6). Figure 3C shows that the GST-Etk-SH2 protein can still pull down ARY307F, Y346F, and Y534F mutants but not the ARY551/552F mutant, suggesting that Y551/552 phosphorylation may be involved. It is well known that the SH2 domains of non–receptor tyrosine kinases preferentially bind to their substrates to enhance catalytic activity and substrate recognition (13). Therefore, we scanned the potential phosphorylation sites of AR using the Motif Scan program (http://scansite.mit.edu/motifscan_seq.phtml; ref. 23) and identified that Y551/552 may be a preferred phosphorylation site.
site induced by the Tec family kinases. This is supported by the observations that Etk can still induce tyrosine phosphorylation of the ARY534F mutant and Y551/552F mutation diminished tyrosine phosphorylation of AR induced by Etk (Fig. 3D). These data suggest that Etk may be able to phosphorylate AR at Y534 and Y551/552, and the interaction between the Etk SH2 domain and phosphorylated ARY551/552 may promote their association.

Figure 2. Etk is upregulated in hormone-resistant prostate cancer and induces AR tyrosine phosphorylation. A, the mean scores and frequency of the immunoreactivity with anti-Etk or anti-pARY534 antibodies detected in hormone-naïve (HN), and hormone-resistant human prostate tumor arrays. *, P < 0.001, compared with hormone-naïve prostate tumor. B, representative fields of the tumor arrays. Anti-AR was used as a control. Low (×20)– and high (×400)–magnification views of Etk, pARY534, and AR expression in hormone-naïve and hormone-resistant prostate tumor. C, Etk is associated with AR and induces AR tyrosine phosphorylation in COS-1 cells. COS-1 cells were cotransfected with the indicated plasmids. At 24 h posttransfection, cells were serum starved overnight and then lysed. The association between Etk and AR was determined by immunoprecipitation with anti-AR antibody and followed by immunoblotting with anti-Etk antibody. The effects of Etk on AR tyrosine phosphorylation were examined by immunoprecipitation with anti-AR antibody and followed by immunoblotting with anti-phosphotyrosine (pY) antibody. The levels of Etk or AR in the total cell lysates were monitored by immunoblotting with anti-Etk and anti-AR antibody, respectively. D, overexpression of Etk in mouse prostate induces AR tyrosine phosphorylation. Mouse prostates were dissected from the Etk transgenic and their WT littermate mice, respectively. One half of the dorsolateral prostate lobes were fixed in 10% formalin and subjected to immunohistochemical staining with anti-phosphorylated ARY534 antibody (left). The lysates of the other half gland were subjected to immunoblotting with the antibodies as indicated (center). The intensity of pARY534 and AR in each lane was quantified by using the software Quantity One and the change of pARY534 was normalized with AR (right). The value in lane 1 was set as 1.
Etk stabilizes AR and promotes its activity under androgen-depleted conditions

We previously showed that overexpression of Etk in LNCaP and mouse prostate increases AR protein level (6); however, this change was unlikely due to transcriptional regulation as our quantitative RT-PCR analysis did not detect appreciable change of AR mRNA when Etk was overexpressed or knocked down in LNCaP cells (Fig. 4C). It is

**Figure 3.** Castration promotes association between Etk and AR. A, association between Etk and AR in xenograft tumors is increased at postcastration. Hormone-sensitive and hormone-resistant LNCaP and LAPC4 xenograft tumors were derived as described in Materials and Methods. The total protein lysates extracted from these tumors were subjected to immunoprecipitation with anti-AR or anti-Etk antibody and followed by immunoblotting with anti-Etk or anti-AR antibody. Expression of Etk or AR was monitored by immunoblotting of the total cell lysates. Actin was used as a loading control. The intensity of AR-bound Etk or Etk-bound AR in each lane was quantified by using the software Quantity One and normalized with AR or Etk protein level (right). The value of HS LNCaP was set as 1. B, COS-1 cells were transfected with Flag-tagged AR. Cell lysates were incubated with GST-Etk WT or GST-Etk mutants bound to glutathione-Sepharose 4B beads at 4°C for 1 h. The beads were washed intensively, and the bound proteins were detected by immunoblotting with an anti-Flag antibody. GST fusion proteins were visualized with Coomassie blue staining (CBS). C, COS-1 cells were transfected with Flag-tagged AR WT or AR mutants. Cell lysates were incubated with GST-EtkSH2 bound to glutathione-Sepharose 4B beads at 4°C for 1 h. The beads were washed intensively, and the bound proteins were detected by immunoblotting with an anti-Flag antibody. The GST fusion proteins were detected with anti-GST. D, effects of Y534F and Y551/552F mutation on Etk-induced AR tyrosine phosphorylation. AR or AR mutants were cotransfected with Etk or the vector control into COS-1 cells. At 48 h posttransfection, cell lysates were subjected to immunoprecipitation with anti-AR and followed by immunoblotting with anti-phosphotyrosine (pY). The expression of AR and Etk in total cell lysates was monitored by immunoblotting with the indicated antibody. The intensity of phosphorylated AR and AR in each lane was quantified by using the software Quantity One, and the change of tyrosine phosphorylation was normalized with AR (right). The value of phosphorylated AR WT was set as 100%. 

**Etk stabilizes AR and promotes its activity under androgen-depleted conditions**

We previously showed that overexpression of Etk in LNCaP and mouse prostate increases AR protein level (6); however, this change was unlikely due to transcriptional regulation as our quantitative RT-PCR analysis did not detect appreciable change of AR mRNA when Etk was overexpressed or knocked down in LNCaP cells (Fig. 4C). It is
possible that regulation of AR expression by Etk may be through a mechanism at the posttranscriptional level. Therefore, we investigated whether the association with Etk could stabilize AR. Figure 4A shows that overexpression of Etk in LNCaP cells leads to an increase in the half-life of AR protein from 1.8 to 4 hours. On the other hand, knocking down endogenous Etk expression by a specific shRNA in CWR-R1 cells led to destabilization of AR protein (Supplementary Fig. S4). Because ARY551/552 phosphorylation is necessary for the association between Etk and AR, we next asked whether the effect of Etk on AR stability is mediated through ARY551/552. As expected, the half-life of the ARY551/552F mutant was reduced to 2 hours compared with the AR WT ($T_{1/2} = 4$ hours) in Etk-overexpressing cells (Fig. 4B). These results suggest that Etk is associated with AR and stabilizes AR in a manner dependent on the integrity of ARY551/552. Mdm2 was previously shown to regulate AR protein turnover in prostate cancer cells (24); therefore, we tested whether Etk could modulate the interaction between AR and Mdm2. As shown in Fig. 4C, overexpression of Etk with AR in COS-1 significantly diminished the association of AR with endogenous Mdm2, whereas the kinase-inactive EtkKQ mutant had little effect. We further examined the effects of Etk on AR-regulated genes in LNCaP cells. PSA and POV-1/SLC43A1 are well-established AR-regulated genes. Using quantitative RT-PCR, we found that overexpression of Etk significantly increased PSA, and POV-1 mRNA level and knockdown of Etk by specific shRNA decreased the level of these transcripts (Fig. 4D, left). Western blot analysis confirmed that Etk might modulate AR activity at least in part through regulation of the steady-state level of AR protein (Fig. 4D, right).

Because Etk expression was increased after androgen ablation in mouse and human prostates, we were interested in examining whether Etk expression is required for growth of hormone-resistant prostate cancer cells. As shown in Fig. 5A and B, knockdown of Etk expression in hormone-resistant C-81 or CWR-R1 cells by shRNA specific for Etk inhibited growth of C-81 or CWR-R1 cells in androgen-depleted

![Figure 4](image_url)

**Figure 4.** Etk increases AR stability and transcriptional activity. A, LNCaP cells were transfected with control vector or Etk. After serum starvation, cells were treated with 0.1 μg/μL cycloheximide (CHX) for the indicated time. Total cell lysates were immunoblotted with anti-AR, anti-Erk, and anti-Etk, respectively. Actin was used as a loading control. B, AR WT or ARY551/552F was cotransfected with Etk into COS-1 cells. After serum starvation, cells were treated with 0.1 μg/μL cycloheximide for the indicated time. Total cell lysates were immunoblotted with anti-AR and anti-Etk. C, AR was cotransfected with T7-tagged Etk or EtkKQ into COS-1 cells. Association of AR with endogenous Mdm2 was detected by immunoprecipitation with anti-Mdm2, followed by immunoblotting with anti-AR. D, LNCaP cells were transfected with lentivirus encoding Etk, shRNA specific for Etk, or vector control. PSA, POV1, and AR mRNA level were detected by quantitative real-time RT-PCR after serum starvation overnight. **, $P < 0.01$. PSA, AR, and Etk protein levels were detected by Western blot.
Similar growth inhibition effects were also observed in xenograft models. These data suggest that Etk is required for growth of C-81 or CWR-R1 both \textit{in vitro} and \textit{in vivo}. To further test whether Etk promotes castration-resistant tumor growth in the xenograft models, Etk or a control vector was transfected into LNCaP cells and then s.c. injected into castrated SCID mice. Figure 5C shows that growth of Etk-overexpressing cells was significantly increased compared with control. Taken together, these results suggest that overexpression of Etk may promote castration-resistant tumor growth.

**Discussion**

The transition from hormone-sensitive to hormone-resistant state after androgen ablation therapy is a major obstacle in effective control of prostate cancer. Multiple mechanisms may be underlying this transition. In the present study, we showed that tyrosine kinase Etk is upregulated in prostate cells in response to androgen deprivation and may play a role in castration-resistant progression of prostate cancer. This is supported by our observations that the association between Etk and AR is increased after castration and Etk induces tyrosine phosphorylation of AR, leading to an increase in AR stability and transcriptional activity under androgen-depleted conditions. In addition, knockdown of Etk expression by specific shRNA leads to attenuation of prostate tumor growth under androgen-depleted conditions, whereas overexpression of Etk promotes castration-resistant growth of prostate tumor xenografts. Therefore, Etk may be a component of the adaptive compensatory mechanism that protects prostate cells from androgen ablation and promotes prostate tumor growth, at least in part, through modulation of AR signaling pathway. It should be noted that Etk is also upregulated in normal mouse prostate in response to castration but is unable to stabilize AR. It is possible that additional changes in prostate tumors may facilitate the effects of Etk on regulation of AR stability.

Most castration-resistant prostate cancers continue to express AR at levels similar to androgen-dependent prostate...
cancers, which indicates that AR still retains its function in castration-resistant prostate cancer. The persistence of AR in prostate cancer independent of circulating androgen levels suggests that AR is activated and thus stabilized by low levels of androgens or other ligand-independent mechanisms. It is known that several growth factors such as epidermal growth factor/transforming growth factor-α, IL-6, and insulin-like growth factor 1, and their downstream tyrosine kinases such as erbB2, Src, and FAK, can activate AR and minimize or possibly even negate the requirement for ligand (18, 25–32). In the present study, we found that Etk, a downstream effector of PI3K, Src, and FAK, is dramatically upregulated in normal mouse prostates and human prostate tumors after androgen deprivation and stabilizes AR through posttranslational modifications. Although the magnitude of AR phosphorylation induced by EtkWT was not as dramatic as that induced by an active Src mutant, AR Y534 phosphorylation detected in human prostate tissues seems to have a stronger correlation with the level of Etk kinase (Pearson correlation coefficient $r = 0.71$, $P < 0.0001$) than that with phosphorylated Src Y416 (Pearson correlation coefficient $r = 0.33$, $P < 0.0001$) reported in our previous study (6). It is likely that detected AR Y534 phosphorylation in prostate tissues may be partly attributed to Etk kinase activity. The relative contribution of each kinase to the regulation of AR activity may depend on cellular context. It is possible that in cells with elevated Src activity, Src may play a major role as Src seems to be a stronger Y534 kinase. However, in those cells in which Src activity is not elevated, Etk could be activated through different mechanisms, such as through overexpression of PI3K/FAK pathways, and could become the key kinase for Y534. Such a possibility is supported by a strong correlation between Etk expression and AR phosphorylation level and Y534 phosphorylation in human prostate tumors. It is possible that Etk kinase might be activated by some stimuli (e.g., IL-6, neuropeptides, matrix proteins, reactive oxygen species) upregulated in prostate tissues upon androgen deprivation. In addition to Y534, Etk may also induce phosphorylation of Y551/552 embedded in the preferred substrate sequence for Tec family kinases. This is supported by the diminished tyrosine phosphorylation of the AR Y551/552 mutant compared with AR WT. Mutation of Y551/552 residues disrupted association of AR with the SH2 domain of Etk, suggesting that the interface between AR and Etk may be very different from that between AR and Src, which is believed to involve the proline-rich region of AR and the SH3 domain of Src (33). This possibility is supported by our observation that Etk-induced Y534 phosphorylation is dependent on the integrity of Y551/552, whereas Y551/552F mutation has little effect on Src-induced Y534 phosphorylation (Supplementary Fig. S5). It is still not clear whether these proteins are present in a common, independent, or sequential signaling complex. Regulation of AR activity by multiple kinases may allow fine-tuned control of its signaling, temporally and spatially in response to diverse extracellular stimuli.

In addition, Etk expression is differentially regulated by androgen and IL-6 in prostate cells, and AR is differentially recruited to the AREs located in the ETK locus, suggesting that Etk may serve as an integrator of both steroid hormone and growth factor signaling. It is yet to be determined how differential regulation of ETK by androgens and IL-6 occurs in prostate cells. It is possible that AR may be associated with different protein complexes or cooperate with different transcription factors in prostate cancer cells in response to various stimuli. Further experiments are necessary to investigate detailed mechanisms by which Etk is regulated by AR.

Taken together, our findings suggest that compensatory upregulation of Etk in response to androgen derivation may promote castration-resistant growth of prostate cancer, at least partially through modulation of AR signaling pathway, and Etk may potentially serve as a diagnostic marker and drug target for prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

NIH grant CA106004, Department of Defense (DOD) grant W81XWH-06-1-0174 (Y. Qiu), NIH grant 1U01-CA86772 (J. Melamed), and DOD Pre-doctoral Fellowship (W81XWH-08-1-0068; D.E. Linn).

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 12/18/2009; revised 04/12/2010; accepted 04/27/2010; published OnlineFirst 06/22/2010.

References


www.aacrjournals.org

Cancer Res; 70(13) July 1, 2010


Compensatory Upregulation of Tyrosine Kinase Etk/BMX in Response to Androgen Deprivation Promotes Castration-Resistant Growth of Prostate Cancer Cells


Cancer Res 2010;70:5587-5596. Published OnlineFirst June 22, 2010.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-09-4610

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2010/06/22/0008-5472.CAN-09-4610.DC1

Cited articles
This article cites 33 articles, 21 of which you can access for free at:
http://cancerres.aacrjournals.org/content/70/13/5587.full#ref-list-1

Citing articles
This article has been cited by 11 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/70/13/5587.full#related-urls

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.