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Disruption of ETV6 leads to TWIST1-dependent progression and resistance to epidermal growth factor receptor tyrosine kinase inhibitors in prostate cancer

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Abstract

Background: ETS variant gene 6 (ETV6) is a putative tumor suppressor and repressed by epidermal growth factor receptor (EGFR) signaling in prostate cancer. Since EGFR antagonists seem ineffective in castration-resistant prostate cancer (CRPC), we aim to study the role of ETV6 in the development of drug resistance.

Methods: ETV6 target gene was validated by CHIP and promoter reporter assays. Correlation of ETV6 and TWIST1 was analyzed in human clinical datasets and tissue samples. Migration, invasion, and metastasis assays were used to measure the cellular responses after perturbation of ETV6-TWIST1 axis. Proliferation and tumor growth in xenograft model were performed to evaluate the drug sensitivities of EGFR-tyrosine kinase inhibitors (TKIs).

Results: ETV6 inhibits TWIST1 expression and disruption of ETV6 promotes TWIST1-dependent malignant phenotypes. Importantly, ETV6 is required to the anti-proliferation effects of EGFR-TKIs, partly due to the inhibitory function of ETV6 on TWIST1. We also found that EGFR-RAS signaling is tightly controlled by ETV6, supporting its role in TKI sensitivity.

Conclusions: Our study demonstrates that disruption of ETV6 contributes to EGFR-TKI resistance, which is likely due to derepression of TWIST1 and activation of EGFR-RAS signaling. Our results implicate ETV6 as a potential marker for predicting efficacy of an EGFR-targeted anticancer approach. Combination treatment of TWIST1 inhibitors could sensitize the anti-proliferation effects of EGFR-TKIs.

Keywords: ETV6, TWIST1, EGFR, TKI

Background

ETS variant gene 6 (*ETV6*), which belongs to the E26 transformation-specific (ETS) transcription factor family, was suggested to play a role as a tumor suppressor due to observed deletions in last stage, advanced prostate cancer [1–4]; however, its biological functions remain unclear. We demonstrated that *ETV6* exhibits antitumor effects suppressing proliferation and metastatic progression, and

found that epidermal growth factor receptor (EGFR) signaling inhibits *ETV6* through a microRNA-mediated mechanism [5]. EGFR signaling is involved in prostate cancer progression [6–9]; however, single-agent therapy using an EGFR tyrosine kinase inhibitor (TKI) was ineffective in castration-resistant prostate cancer (CRPC) [10]. Since resistance to EGFR antagonists eventually develops and remains a challenging phenomenon [11], it is possible that loss of *ETV6* function promotes the development of drug resistance in prostate cancer.

EGFR signaling follows three general steps: a ligand-monomeric EGFR interaction, dimerization (either homodimer or heterodimer) coupled with autophosphorylation through tyrosine kinase activity, and signal transduction for tumor-related properties [11]. Therefore, mutations

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altering either the EGFR structure or activities of downstream components (e.g., *KRAS*) are recognized as key mechanisms responsible for resistance. However, several studies showed that the epithelial-to-mesenchymal transition (EMT) also plays a critical role in drug resistance consistent with the cancer-stem-cell (CSC) hypothesis [12, 13]. Indeed, the EMT-based gene profile is a powerful predictor of resistance to EGFR inhibitors [14]. Also, one important transcription factor of the EMT, *TWIST1* [15], was found to be associated with EGFR activation [16–19] and attributable to drug resistance [20–22]. In prostate cancer, Twist family BHLH transcription factor 1 (*TWIST1*) was found to be highly expressed in 90% of prostate cancers compared to 6.7% of benign hyperplasia [22] and is involved in the development of CRPC [23]. Consistent with the CSC hypothesis, *TWIST1* promotes tumor sphere formation, a functional indication of the self-renewal ability and CSC population [21].

Both *PTEN* and *TP53* are frequently disrupted in prostate cancer; in addition, *TP53* mutations occur in half of all CRPC [24, 25]. Following our earlier studies of *ETV6* [5], we continued to investigate the molecular mechanism underlying its antitumor effects by utilizing prostate cancer cells derived from a prostate-specific *Pten/Trp53* double-knockout mouse [24, 26]. We demonstrated that *Etv6* associates at the promoter region of *Twist1* and suppresses its transcription in a sequence-dependent manner. In human prostate cancer cells, *ETV6* also inhibits *TWIST1* expression and *ETV6*-knockdown can promote *TWIST1*-dependent malignant phenotypes. Importantly, perturbation of *ETV6*-*TWIST1* axis can contribute to development of drug resistance. Prostate cancer cells with *ETV6*-knockdown are insensitive to TKIs while exogenous expression of *ETV6* restores the anti-proliferative effects in the TKI-resistant RasB1 cell line, which expresses a mutated RAS oncogene [27, 28]. We also found an inhibitory circuit between *ETV6* and EGFR-RAS signaling; therefore, there could be multiple mechanisms accounting for the drug-sensitizing effect of *ETV6*. Our results provide a molecular mechanism by which *ETV6* suppresses tumor progression through transcriptional regulation of *TWIST1* and disruption of EGFR-RAS signaling.

Methods

Cells, constructs, and reagents

The mouse AC1, AC3, C1, and C2 cell lines were isolated from $PbCre4^{+};Pten^{fl/fl};TP53^{fl/fl}$ Luc + mouse prostate tumors and were established as previously described [24, 26]. AC1 and AC3 cells were cultured in PrEGM medium (Lonza, Walkersville, MD, USA); C1 cells were cultured in PrEGM/DHT with 5% serum and 5% 3 T3-conditioned medium; C2 cells were cultured in PrEGM/

DHT with 5% 3 T3-conditioned medium. The mouse wild-type (WT) prostatic basal cell line was provided by Dr. Lei Fang (NCI/NIH, Bethesda, MD, USA) and was cultured in WIT-P medium (Stemgent, San Diego, CA, USA) as previously described. DU145, PC3, LNCaP, and 22RV1 human prostate cancer cell lines were obtained from ATCC (Rockville, MD, USA). The metastatic RasB1 cell line was previously characterized and used to study molecular mechanisms of prostate cancer metastasis in multiple peer-reviewed articles [27–33]. All human prostate cancer cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). RasB1 and PC3 cells with stable expression of *ETV6* were established by transfection with an *ETV6* complementary (c)DNA-encoding or empty pCDH-CMV-MCS-EF1-Puro vector (System Biosciences, Palo Alto, CA, USA); 2×10^5 cells were seeded and transfected with 5 μ g DNA and selected with puromycin for 1 month. Mouse and human ON-TARGETplus SMART-pool siRNAs (scrambled and *ETV6*) and a human shRNA vector (LacZ and *ETV6*) were from Dharmacon (Thermo Scientific, Waltham, MA, USA) and the RNAi Core Lab (Academia Sinica, Taipei, Taiwan), respectively. Transient transfections of plasmids and siRNAs were carried out using the X-tremeGENE HP DNA transfection reagent (Roche, CA, USA) or Lipofectamine RNAi-MAX (Invitrogen, Carlsbad, CA, USA). Cells were treated with EGFR inhibitors, CI1033 (10 ng/ml) and AG1478 (10 μ M) for 24 h in medium containing 10% serum. For EGF treatment, cells were subjected to serum-starvation for 24 h, followed by the addition of 100 ng/ml EGF for 24 h also in serum-free medium. The EGF was from R&D Systems (Minneapolis, MN, USA), and the EGFR inhibitors (CI1033 and AG1478) were from Selleck (Houston, TX, USA). The mouse *Etv6*-binding site was located upstream of mouse *Twist1* on chromosome 12: 33957354 at GRCm38. The *Twist1*-red fluorescent protein (RFP) reporter containing the mouse *Twist1* promoter with the *Etv6* response element was constructed using a Clone-it Enzyme free Lentivectors Kit (System Biosciences). *ETV6* response element mutations were made using a Site-Directed Mutagenesis System kit (Invitrogen). All primers used for these constructs are listed in Additional file 1; Table S1. All constructs were verified by a DNA sequence analysis.

Quantitative real-time reverse-transcription (qRT)-polymerase chain reaction (PCR)

An qRT-PCR was used to measure *Etv6*, *Cdh1*, *Twist1*, and *Vim* in mouse cell lines or *ETV6* and *TWIST1* expressions in human prostate cancer cell lines. Total RNA was isolated using the mirVana PARIS RNA isolation system (Thermo Scientific, Waltham, MA, USA). For RT, 3 μ g of total RNA was used with the SuperScript

III kit (Invitrogen). Samples containing primer pairs were mixed in SYBR green PCR master mix (Applied Biosystems, Waltham, MA, USA), and the amplification program was as follows: initial 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were normalized to mouse *Gapdh* or human *GAPDH* expression and run in triplicate. All primers used for the PCR are listed in Additional file 1; Table S2.

Chromatin Immunoprecipitation (ChIP) assay

ChIP assays were performed using the EZ magna ChIP A kit (Millipore, Billerica, MD, USA) with a modified protocol. For small interfering (si)RNA treatment, 10^7 AC1 cells in 10-cm dishes were transfected with mouse scrambled or ETV6 siRNAs for 48 h. Cells were cross-linked with 1% formaldehyde in culture medium at room temperature for 15 min and then quenched with the addition of 1 ml of 10× glycine. Cells were washed twice with cold phosphate-buffered saline (PBS) containing a protease inhibitor (Roche) and centrifuged at 10^5 rpm. Cell pellets were resuspended in 0.5 ml of cell Lysis Buffer (BioRad, Hercules, CA, USA) and incubated on ice for 15 min. Nuclei were collected by centrifugation at 10^5 rpm and 4 °C for 10 min and resuspended in nuclear lysis buffer. Genomic DNA was sheared by a microtip during sonication (Branson Sonifier 250, Germany) following 15 cycles of a 20-s burst then 1 min of cooling on ice. This procedure resulted in DNA fragments sized approximately 100~300 bp. Sheared chromatin was aliquoted to perform immunoprecipitation with a control immunoglobulin G (IgG) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or antibodies against ETV6 or *Gapdh* at 4 °C overnight. A qPCR was performed in triplicate with 2 µl of eluted chromatin. ChIP antibodies and PCR primers are listed in Additional file 1; Table S3.

Promoter reporter assay

For promoter reporter assays, AC1 or AC3 cells in 12-well plates (5×10^4 cells/well) were transiently transfected with 1 µg of the mouse *Twist1*-RFP reporter containing the ETV6 response element (RE). Cells were also pretreated with 100 nM siRNA (scrambled, mouse ETV6) or 1 µg DNA (empty vector or ETV6-expressing vector, OriGene, Rockville, MD, USA) by transfection. The promoter function was analyzed using fluorescence-activated cell sorting (FACS, BD Biosciences, San Jose, CA, USA), and relative median fluorescent intensity (MFI) values were measured as previously described [28]. The MFI value for the RFP was measured by FACS using FACSDiva software (BD Biosciences) and was normalized to the value of the vehicle. Three independent experiments were run with triplicate samples.

Correlation analyses using human gene expression datasets

To compare ETV6 expression levels with prostate cancer progression and with TWIST1 expression levels, we used mRNA expression data from human prostate cancer databases of the Taylor dataset [3] and the Cancer Genome Atlas (TCGA). The study using the Taylor dataset was conducted under Memorial-Sloan Kettering Cancer Center (MSKCC) Institutional Review Board approval on 98 primary and 13 metastatic prostate cancer samples in addition to 28 normal prostate samples. Analysis of TCGA dataset was performed with 48 normal solid tissues.

Western blot analysis

Cells grown on 6-well plates (10^6 cells/well) were lysed in 150 µl RIPA buffer containing complete protease inhibitors (Roche) and phosphatase inhibitors (Roche), 25 mM β-glycerophosphate, 10 mM sodium fluoride, and 1 mM sodium vanadate. Twenty micrograms of protein was separated per lane by sodium dodecylsulfate (SDS)-gel electrophoresis. After being transferred to polyvinylidene difluoride membrane, blots were blocked with 5% BSA in PBST. Primary antibodies were incubated overnight at 4 °C, and secondary antibodies were incubated at room temperature for 1 h as indicated in Additional file 1; Table S4.

Tissue samples

Twenty-two cases of prostatic adenocarcinoma were collected from the Taipei Medical University Joint human biological database (Taipei, Taiwan), and approved by the Taipei Medical University-Joint Institutional Review Board (approval no.: 201311034). RNA was extracted from dissected tissue containing greater than 70% tumor cell content. The method for separating the specimens into two groups of 'low' (TWIST1_L) and 'high' TWIST1 (TWIST1_H) expressions was pre-decided by half of the number of patients according to TWIST1 levels by an RT-qPCR.

Migration and invasion assay

For the migration and invasion assay, metastatic RasB1 and PC3 cells were stably transfected with the ETV6 expression vector or an empty vector. DU145 cells were stably transfected with a LacZ or ETV6 shRNA vector. LNCaP and 22RV1 cells were transiently transfected with SMARTpool ETV6 siRNA or control scrambled siRNA. Cells were resuspended at a concentration of 2.5×10^5 cells/ml in serum-free medium. Matrigel™ for the invasion assay was purchased from BD Biosciences (San Jose, CA, USA). Matrigel-coated transwell dishes were prepared by adding 200 µl of 10-fold serum-free medium-diluted Matrigel. In total, 2.5×10^5 cells/well in serum-free medium was plated above the Matrigel. The

lower chamber was filled with 600 μ l of serum-containing medium or serum-free medium with the addition of 200 ng/ μ L EGF. Cells that had invaded the Matrigel-coated transwells in response to EGF after 12 h were fixed and stained with a 0.5% crystal violet fixative solution for 15 min. Invaded cells on the underside of the membrane were counted and quantified by an enzyme-linked immunosorbent assay (ELISA) reader at OD 550 nm for each replicate in triplicate. The migration assay used transwells without Matrigel, and cells were fixed and stained as described for the invasion assay.

Animal studies

Animal work was performed in accordance with a protocol approved by the Taipei Medical University Animal Care and Use Committee (Taipei, Taiwan). For the metastasis analysis, DU145 cells harboring a LacZ or ETV6 shRNA vector were subjected to intracardiac injections into 5-week-old male nude mice (National Laboratory Animal Center, Taipei, Taiwan; six mice/group) at 10^5 cells per mouse. For survival studies, mice were euthanized when one of the following situations applied: 10% loss of body weight, paralysis, or head tilting. Hematoxylin and eosin (H&E) staining of brain tissues of mice was performed on day 80 after the injection as previously described [30]. To analyze tumorigenesis, 5-week-old male nude mice (National Laboratory Animal Center; five mice/group) were subcutaneously injected with 10^6 RasB1 cells harboring an empty or ETV6 expression vector in 50% Matrigel™ (BD Biosciences). Subcutaneous tumors were harvested and measured from mice after treatment with 20 mg/kg CI1033 or DMSO as the control for 1 month as previously described [28].

Proliferation assay

DU145 cells were stably transfected with a LacZ or ETV6 or TWIST1 short hairpin (sh)RNA vector, or RasB1 cells were stably transfected with an empty or ETV6 or TWIST1 expression vector, and seeded at a density of 2×10^3 cells/well in 96-well plates. Cells were treated with 0, 0.1, 0.5, 1, 2, 5, and 10 nM CI1033 or 0, 0.1, 0.5, 1, 2, 5, and 10 μ M AG1478 for 24 h, and analyzed using a Cell Proliferation Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Statistical analysis

All data are presented as the mean \pm standard error of the mean (SEM). Statistical calculations were performed with GraphPad Prism analytical tools. Differences between individual groups were determined by Student's *t*-test or a one-way analysis of variance (ANOVA) followed by Bonferroni's post-test for comparisons among three or more groups. The association between ETV6 and TWIST1

expressions was compared using a Chi-squared test. *p* values of < 0.05 were considered statistically significant.

Results

Etv6 recognizes the promoter of Twist1 and suppresses its transcription

When investigating the role of ETV6 in tumor progression, we hypothesized that ETV6 was inhibitory to the EMT. Our earlier study concluded that loss of *Pten* and *TP53*, two common genetic lesions in prostate cancer, promotes the EMT and cell plasticity in a mouse prostate-specific *Pten/Trp53* double-knockout mouse model [24, 26]. When comparing the expression between one clonally derived cell line from this model and that of normal mouse prostate tissues, many EMT transcription factors were increased, while *Etv6* was comparatively reduced (Additional file 1; Figure S1A). We further investigated a panel of mouse prostate cancer cell lines derived from the mouse model (Fig. 1A), and specifically analyzed the expression pattern of *Twist1* due to its association with prostate cancer [22, 23]. Consistent with our hypothesis, *Etv6* was positively correlated with an epithelial marker (*Cdh1*) (Pearson correlation, $r = 0.966$) but negatively with *Twist1* (Pearson correlation, $r = -0.9002$) (Fig. 1B). We further focused on a comparison between AC3 and AC1, since AC3, but not AC1, exhibits the transforming growth factor (TGF)- β -induced EMT [26]. Again, both *Etv6* and *Cdh1* were reduced, while the mesenchymal markers (*Twist1* and *Vim*) were increased in AC3 (Fig. 1C). In summary, our results showed that *Etv6* is negatively associated with the EMT.

Because *Etv6* is a transcription repressor [34], we searched for a consensus sequence on the promoter of *Twist1* and identified one *Etv6* response element (RE) at -1286 (Fig. 1D). We performed chromatin immunoprecipitation (ChIP) assays and found an enriched signal at the *Etv6* RE only when using an antibody against *Etv6*, but not the antibody against *Gapdh*, suggesting that the signal was specific to *Etv6* (Fig. 1E). When using the same *Etv6*-specific antibody, we observed no enrichment at a site containing no *Etv6*-consensus sequence (Non-*Etv6* RE), suggesting that the binding was sequence-specific (Fig. 1E). In addition, the ChIP signal derived from the *Etv6* RE was significantly reduced after *Etv6*-knockdown using *Etv6*-specific siRNA (si*Etv6*), further supporting that binding was *Etv6*-dependent (Fig. 1F). We thus analyzed the cis-effect of the *Etv6* RE on transcription by performing a reporter assay. The reporter activity of the construct containing the WT *Etv6* RE was increased after *Etv6* knockdown (scr. vs. si*Etv6*, Fig. 1G), while it was reduced after expression of exogenous *Etv6* (EV vs. *Etv6*, Fig. 1H). The reporter construct containing a mutated (Mut) *Etv6* RE was not responsive to different

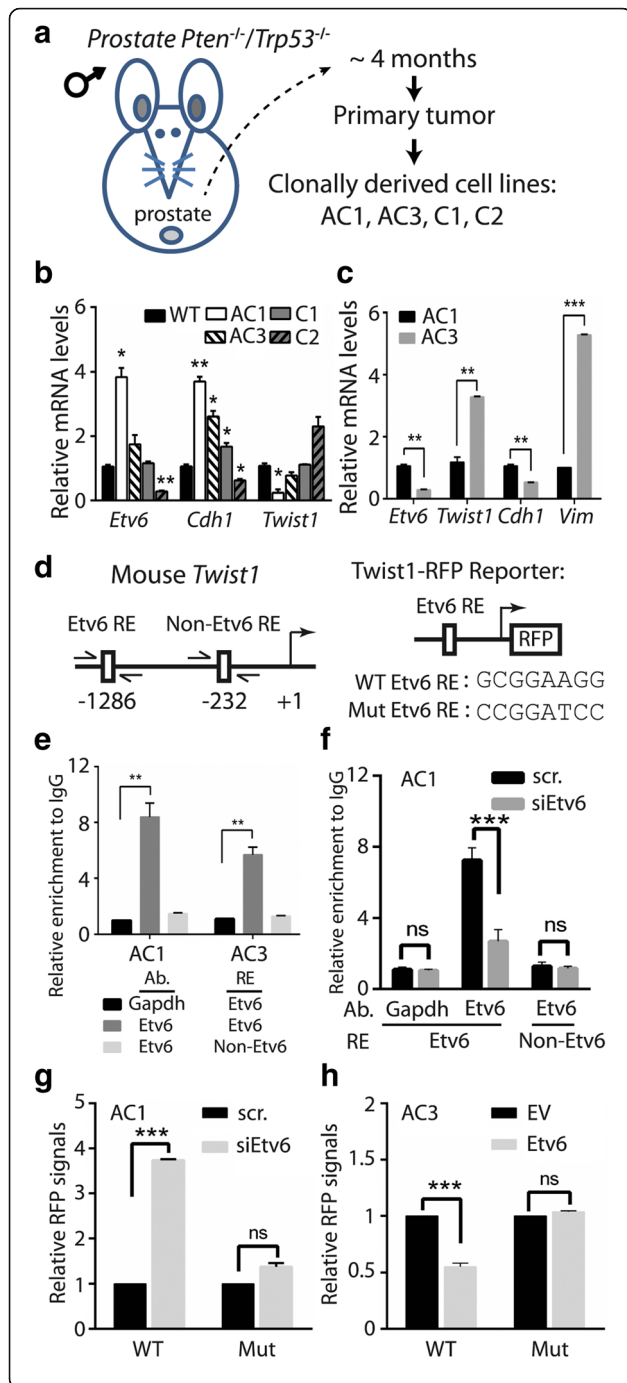


Fig. 1 *Twist1* is transcriptionally suppressed by Etv6. **(a)** Establishment of mouse prostate cancer cell lines (AC1, AC3, C1, and C2) from primary tumors of prostate-specific *Pten/Trp53*-null mice. **(b)** Monitoring of mRNA in a wild-type (WT) mouse prostatic basal cell line and four mouse prostate cancer cell lines. **(c)** Monitoring of mRNA in AC1 and AC3 prostate cell lines. **(d)** Left: Schematic of a predicted ETV6 response element (Etv6 RE) and a non-specific site (non-ETV6 RE) on the mouse *Twist1* promoter. Right: Mouse Twist1-red fluorescent protein (RFP) reporter construct containing the WT or mutated ETV6 response element (WT vs. Mut), followed by the RFP. **(e)** ChIP analysis using an antibody (Ab) against the Etv6 protein at two RE sites (Etv6 vs. non-Etv6) in two mouse prostate cancer cell lines. An antibody against GAPDH at ETV6 RE served as a nonspecific control. The signal was determined as a percentage of the total input and was then normalized to immunoglobulin G (IgG). **(f)** ChIP analysis in response to Etv6 knockdown (siEtv6). scr., control siRNA. **(g, h)** Twist1-RFP reporter assay. The signal of the reporter construct containing either a WT or Mut ETV6 RE was measured in response to Etv6-knockdown (scr. vs. siEtv6, panel **g**) or ETV6 expression by transient transfection (EV vs. Etv6, panel **h**). EV, control vector. Quantification of mRNA was normalized to *Gapdh*, and results are presented as the mean \pm SEM, $n = 3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, non-significant

ETV6 statuses (Fig. 1G, H). These results suggest that ETV6 inhibits the EMT, partly through suppression of *Twist1* by a physical interaction at the promoter of *Twist1*.

ETV6 is inversely correlated with TWIST1 in human prostate cancer

To further confirm the negative relationship between ETV6 and TWIST1 in human prostate cancer, we analyzed two public prostate cancer datasets. Many EMT drivers either showed positive or no significant correlation, instead of a negative one (Fig. 2A); however, only *TWIST1* showed a negative correlation with *ETV6* in both datasets, supporting ETV6-specific regulation (Fig. 2B, C). In addition, in the Taylor prostate dataset [3], mean expression of the *ETV6* gene was significantly lower in primary tumors and further reduced in metastatic tumors compared those in normal tissues (Fig. 2D). On the contrary, the distribution profile was completely reversed with *TWIST1* (Fig. 2E). Consistent with the profile analyzed from the Taylor dataset, both the messenger (m)RNA and protein levels of ETV6 were lower in the metastatic RasB1 cell line than those in non-metastatic cells (22RV1, LNCaP, and DU145) (Fig. 2F, G). Again, *TWIST1* was negatively associated with ETV6 and increased only in metastatic RasB1 cells (Fig. 2F, G). We further investigated the relationship between ETV6 and *TWIST1* in human prostate cancer tissues collected from the Taipei Medical University Joint human biological database (approval no.: 201,311,034, Taipei, Taiwan). We divided samples into two groups based on relative *TWIST1* levels (*TWIST1*_H vs. *TWIST1*_L) and found a negative association between ETV6 and *TWIST1* (Fig. 2H, left panel). An inverse correlation was also demonstrated in

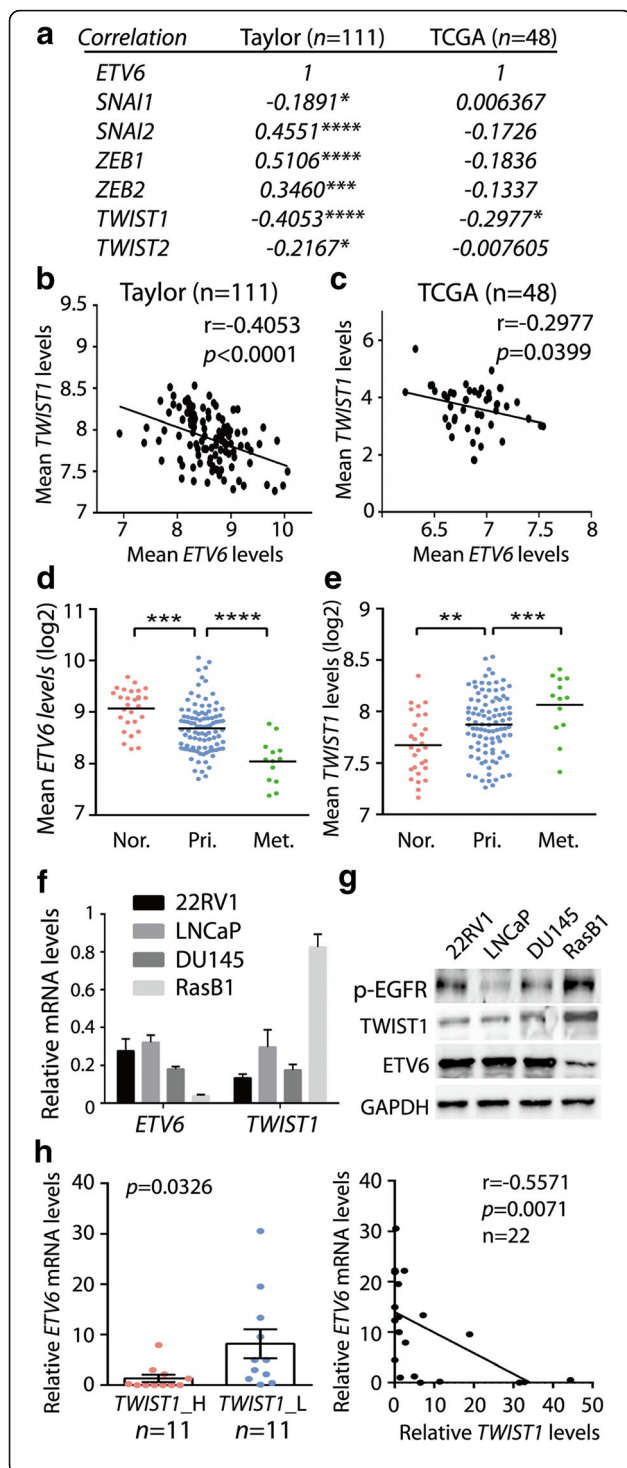


Fig. 2 *ETV6* is inversely correlated with *TWIST1* in human prostate cancer. **(a)** Pearson correlation analysis between *ETV6* and epithelial-to-mesenchymal transition (EMT) transcription factors, using the Taylor and TCGA clinical prostate cancer datasets. **(b, c)** Correlation profiles between *TWIST1* and *ETV6* in the Taylor **(b)** and TCGA **(c)** clinical prostate cancer datasets. **(d, e)** Mean mRNA analysis of *ETV6* **(d)** or *TWIST1* **(e)** in normal prostate tissues (Nor., $n = 28$), and primary (Pri., $n = 98$) and metastatic (Met., $n = 13$) prostate cancer of the Taylor prostate cancer dataset. **(f)** Monitoring *ETV6* and *TWIST1* mRNAs in a panel of human prostate cancer cells. Gene expression was normalized to *GAPDH*. **(g)** Measurement of *ETV6* and *TWIST1* protein in prostate cancer cells by a Western blot assay. **(h)** Quantification of mRNA in prostate cancer tissue samples from the Taipei Medical University Joint human biological database. Left panel: Association analysis between *ETV6* and *TWIST1* in samples with two relative *TWIST1* levels (H, high; L, low, $n = 11$ each group). Right panel: Pearson correlation analysis between *ETV6* and *TWIST1* mRNA in the same set of human prostate samples. Significance was determined by the Gaussian population (Pearson) and a two-tailed test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

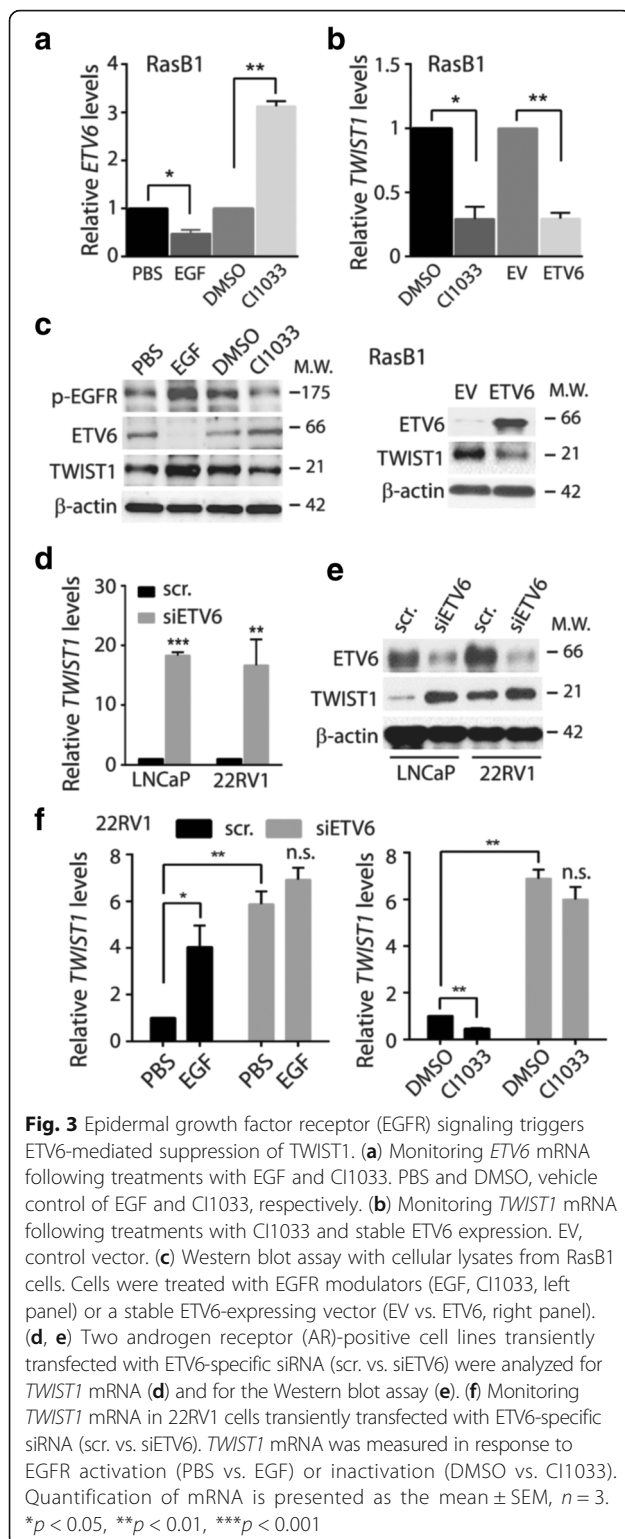
the same set of tissue samples (Fig. 2H, right panel). In summary, we concluded that *TWIST1* is negatively associated with *ETV6* and is involved in tumor progression in human prostate cancer.

ETV6 tightly controls *TWIST1* expression and serves as a mediator of EGFR-*TWIST1* signaling in human prostate cancer cells

ETV6 exhibits antitumor effects and can be negatively regulated by EGFR signaling [5]. Since *TWIST1* was shown to be a downstream effector following EGFR activation [16–19], *ETV6* could be a crucial component in the pathway. To test this possibility, we confirmed that EGFR activity negatively regulated *ETV6* mRNA (Fig. 3A), and that using either an EGFR inhibitor (CI1033) or overexpression of *ETV6* could reduce *TWIST1* mRNA in metastatic RasB1 cells (Fig. 3B). We observed the same effects by monitoring their protein levels (Fig. 3C). In non-metastatic prostate cancer cells, which express more *ETV6* compared to RasB1 (Fig. 2G), *ETV6* knockdown efficiently increased *TWIST1* at both the mRNA and protein levels (Fig. 3D, E), suggesting that *TWIST1* is tightly controlled by *ETV6*. To determine whether *ETV6* is involved in EGFR-*TWIST1* signaling, we found that treatments modulating EGFR activities (i.e., EGF and CI1033) no longer affected *TWIST1* following *ETV6* knockdown (si*ETV6*, Fig. 3F). Our results support a novel EGFR-*ETV6*-*TWIST1* pathway in that *ETV6* serves as a gatekeeper to maintain *TWIST1* at low levels in prostate cancer.

ETV6-knockdown leads to *TWIST1*-dependent malignant progression

We demonstrated that *ETV6* efficiently suppressed metastasis of prostate cancer [5]; however, the underlying



mechanism remained unclear. Based on current findings, decreasing *TWIST1* expression could account for the anti-tumor effects of ETV6. To test this idea, we first confirmed the metastasis-related functions of *TWIST1* by monitoring

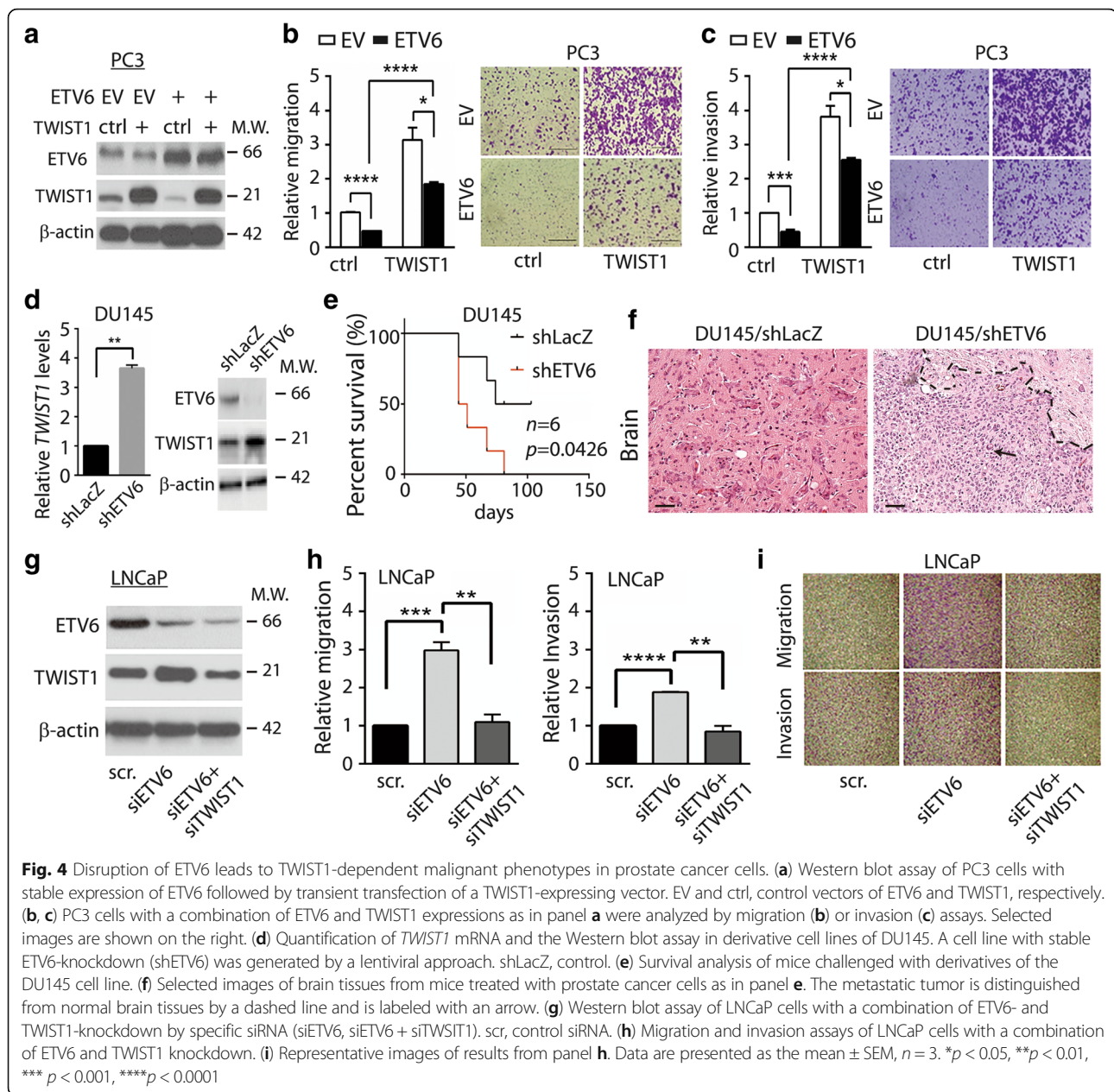
a metastatic PC3 cell line stably expressing exogenous ETV6, followed by transient expression of *TWIST1* (Fig. 4A). Although ETV6 did suppress the malignant abilities (both migration and invasion), overexpression of *TWIST1* clearly enhanced those in the presence of ETV6 (ETV6 vs. ETV6 + *TWIST1*, Fig. 4B, C). We observed the same malignant effects of *TWIST1* when performing these experiments in RasB1 cells (Additional file 1; Figure S1B).

Consistent with the antitumor function of ETV6, stable ETV6-knockdown in DU145 cells efficiently promoted the malignant abilities (shLacZ vs. shETV6, Additional file 1; Figure S1C). Again, we confirmed that *TWIST1* was induced after ETV6-knockdown (Fig. 4D). When simulating the metastasis process by delivering this pair of cells into mice by an intracardiac injection, ETV6-knockdown indeed reduced the life expectancy (Fig. 4E) and promoted metastasis according to tumor masses detected in the brain (arrow, Fig. 4F). Since DU145 was derived from a human prostate adenocarcinoma metastasizing to the brain [35], ETV6 knockdown could reactivate the metastatic properties of DU145 to the brain. To test whether the malignant effects following ETV6-knockdown were due to increased *TWIST1*, we successfully reduced both ETV6 and *TWIST1* by specific siRNAs in a non-metastatic LNCaP cell line (Fig. 4G). Compared to ETV6-knockdown alone, which increased migration and invasion, additional *TWIST1*-knockdown reduced both functions to background levels (siETV6 vs. siETV6 + si*TWIST1*, Fig. 4H, I). We observed the same results when performing experiments using 22RV1 cells (Additional file 1; Figure S1D-F). In summary, we concluded that ETV6-knockdown leads to derepression of *TWIST1* which contributes to tumor progression.

ETV6-*TWIST1* signaling is involved in the development of resistance to EGFR antagonists

The EGFR is a validated target for cancer therapy; however, resistance to EGFR inhibitors eventually evolves [11]. Since EGFR-based therapeutics showed no beneficial effects in prostate cancer [10, 36], it is important to determine whether the ETV6-*TWIST1* axis plays a role in the development of drug resistance. We showed the anti-proliferative effects using one EGFR kinase inhibitor (AG1478) in DU145 cells; however, following ETV6-knockdown, cells became resistant (Fig. 5A). Interestingly, simultaneous knockdown of both ETV6 and *TWIST1* recovered the anti-proliferative effect of the inhibitor (shETV6 + si*TWIST1*, Fig. 5A), consistent with our hypothesis that inducing *TWIST1* after disruption of ETV6 contributes to malignant progression. The same experiment was performed using another EGFR inhibitor (CI1033, Additional file 1; Figure S1G).

The RasB1 cell line is derived from DU145 by introducing a mutant RAS and thus is resistant to EGFR TKIs



[27, 28]. We demonstrated again that an EGFR antagonist (CI1033) did not suppress proliferation of RasB1, but overexpression of ETV6 clearly recovered the anti-proliferative function (EV vs. ETV6, Fig. 5B). However, RasB1 was resistant to CI1033 again after co-expression of both ETV6 and TWIST1, supporting the role of TWIST1 in the development of drug resistance (Fig. 5B). The same conclusion was derived using another TKI (AG1478, Additional file 1; Figure S1H). We also used a mouse xenograft model with the subcutaneous injection of RasB1 cells and tested the effect of ETV6 on CI1033. After the mice developed tumors, the mice were treated with CI1033 for 4 weeks. We found that CI1033 did not

significantly reduce tumors (EV/DMSO vs. EV/CI1033); however, cells expressing ETV6 showed drastic antitumor effects (EV/DMSO vs. ETV6/DMSO, Fig. 5C), consistent with our previous findings [5]. Further CI1033 treatment in mice injected with RasB1 cells expressing exogenous ETV6 completely suppressed tumor growth in three of five mice tested (ETV6/CI1033, Fig. 5C, D).

A mutual inhibition circuit exists between EGFR-RAS signaling and ETV6

In addition to the inhibitory role of ETV6 on TWIST1, we investigated the effect of ETV6 on EGFR-RAS signaling. We found that exogenous ETV6 negatively regulated the

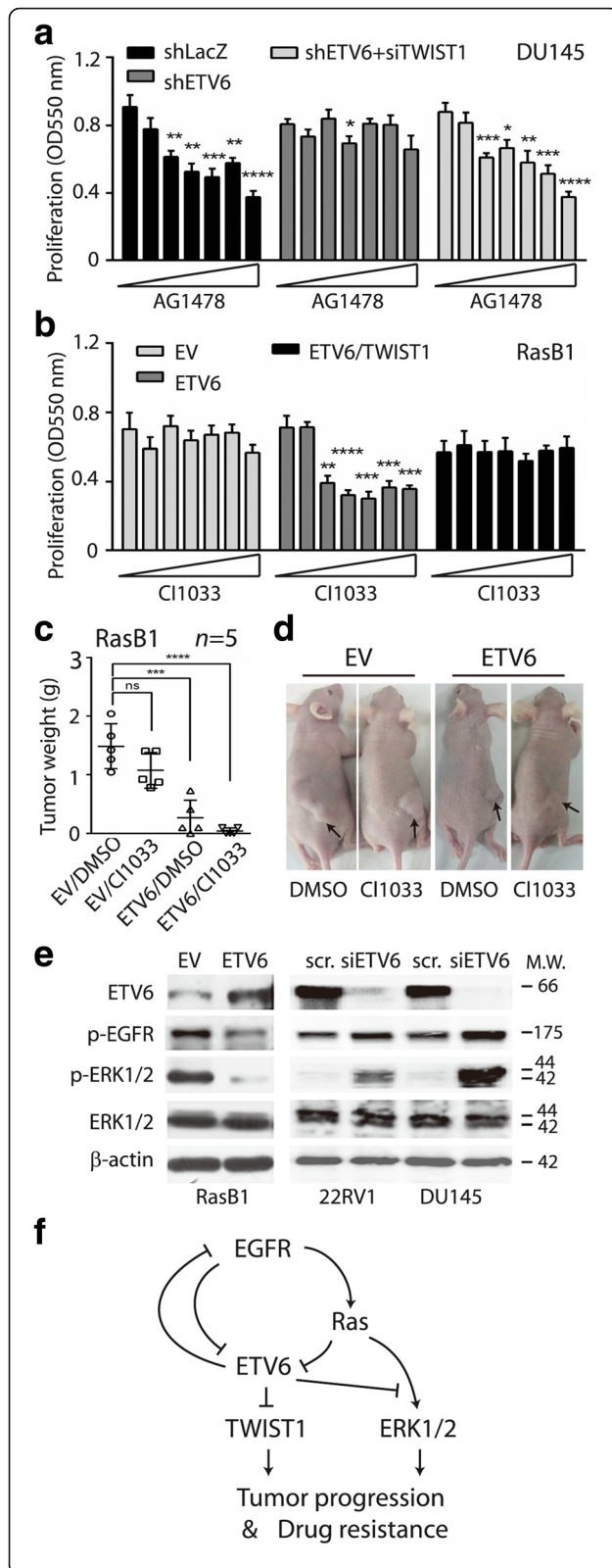


Fig. 5 ETV6-TWIST1 signaling is involved in the molecular mechanism of drug resistance. **(a)** Proliferation assay in three DU145 derived cells treated with a tyrosine kinase inhibitor (TKI: AG1478, 0.1~10 μM), $n = 8$. shLacZ, control; shETV6, ETV6-knockdown; shETV6 + siTWIST1, both ETV6- and TWIST1-knockdown. **(b)** Proliferation assay in three stable RasB1 derived cells treated with a TKI (CI1033, 0.1~10 nM), $n = 8$. EV, control vector; ETV6, ETV6-expressing vector; ETV6 + TWIST1, both ETV6- and TWIST1-expressing vectors. **(c)** Tumor growth analysis of stable RasB1 cell lines (EV vs. ETV6) subcutaneously inoculated in male nude mice followed by treatment with CI1033. Tumor sizes were monitored weekly (left, $n = 5$). At the end, tumor weights were also measured (right, $n = 5$). **(d)** Selected images of mice from panel C, containing tumors (arrows) derived from stable RasB1 cell lines (EV vs. ETV6). **(e)** Western blot analysis of human prostate cancer cells. RasB1 cells were introduced with exogenous ETV6 or under ETV6-knockdown in 22RV1 and LNCaP cells by an siRNA approach (siETV6). EV, empty vector; scr., siRNA control. **(f)** Working model, activation of the epidermal growth factor receptor (EGFR) promotes tumor progression and drug resistance through RAS signaling and suppression of ETV6, which lead to TWIST1-dependent malignant phenotypes. A mutual inhibitory circuit exists between EGFR-RAS signaling and ETV6

phosphorylation status of the EGFR (p-EGFR, Fig. 5E, left) in metastatic RasB1 cells. Interestingly, exogenous ETV6 also efficiently suppressed the phosphorylation status of extracellular signal-regulated kinase 1 and 2 (ERK1/2), a downstream signal transducer of EGFR-RAS signaling [37], while ETV6-knockdown in non-metastatic 22RV1 and DU145 cells increased the phosphorylation signal (p-ERK1/2, Fig. 5E, right). Thus, we hypothesized a mutual inhibition between EGFR-RAS signaling and ETV6. The inhibitory effect of ETV6 on EGFR-RAS signaling could also explain an earlier study showing that ETV6 can suppress RAS-induced transformation in an NIH3T3 cell model [38]. Based on our aggregated results, we propose a working model that disruption of ETV6 contributes to tumor progression and TKI-resistance through derepression of TWIST1 and activation of EGFR-RAS signaling (Fig. 5F).

Discussion

An earlier study showed that the genomic locus containing *ETV6* is the most common translocation site in leukemia [39]. While one allele can fuse to over 30 different genomic loci, the other usually undergoes deletion, which accounts for the frequently observed loss of heterozygosity [38]. Therefore, *ETV6* was considered a tumor suppressor, even in certain types of solid tumors [40]. In prostate cancer, many genes from the ETS family participate in fusion transcripts with transmembrane serine protease isoform 2 (*TMPRSS2*); in fact, three ETS members (*ERG*, *ETV1*, and *ETV4*) contribute to about 80% of *TMPRSS2* fusion [41, 42]. However, *ETV6* has not yet been reported to be involved in chromosome translocation or fusion with *TMPRSS2*; instead, frequent deletions were observed in late stages, metastatic

prostate cancer [1–4]. It was shown that following androgen signaling, both the androgen receptor (*AR*) and topoisomerase II beta (*TOP2B*) were localized to *TMPRSS2-ERG* genomic breakpoints, followed by *TOP2B*-mediated DNA breakage and recombination [43]. Therefore, genomic rearrangements in prostate cancer, although frequently occurring, are likely dependent on the nature of the *AR* and *TOP2B* and are restricted to certain ETS loci except for *ETV6*. Since genomic lesions of *ETV6* are common in leukemia, which does not rely on androgen, deletion of *ETV6* might not be dependent on *AR* signaling.

Androgen deprivation therapy (ADT) is a standard procedure in prostate cancer; however, patients eventually develop metastatic castration-resistant prostate cancer. Therefore, patients could benefit from combining therapeutic approaches with different mechanisms. One would consider *EGFR*-targeted therapy since that the majority of prostate cancers are derived from an epithelial origin and are associated with elevated activities of the *EGFR* family [6–9]; however, using either an *EGFR* antagonist or anti-*HER2* antibody did not achieve therapeutic effectiveness in CRPC [10, 36]. This might reflect the challenging issue for *EGFR*-targeted therapy in general since resistance inevitably occurs even though the antagonists are used in many types of cancer [11]. Our proposed model that disruption of *ETV6* leads to TKI resistance via derepression of both *TWIST1* and *EGFR-RAS* signaling (Fig. 5F), provides one explanation to this issue. Since *ETV6* is frequently deleted in late stage, malignant prostate cancer [1–4], the majority of CRPC could be lack of *ETV6* function, consequently, irresponsive to TKIs. Prostate cancer still at earlier or hormone-sensitive stages containing intact *ETV6* activity could be responsive to *EGFR*-targeted antagonists as monotherapy. Patients might benefit from prescreening their *ETV6* statuses (genetics or expression levels) before treated with *EGFR*-TKIs.

Our results support a model that the *EGFR* facilitates tumor malignancy by reducing *ETV6*, which enhances *TWIST1* activities. In addition, we previously reported that activation of *EGFR* signaling facilitates bone metastasis of prostate cancer through *EGFR*-mediated transcriptional suppression of microRNA-1 (miR-1) [16]. Reduced miR-1 can increase *TWIST1* function since miR-1 targets the 3' untranslated region of *TWIST1* and destabilizes its mRNA [16]. Furthermore, an earlier study also showed that *EGFR* activation can trigger signal transducer and activator of transcription 3 (STAT3)-dependent transcription of *TWIST1* and EMT [17]. Based on conclusions from our studies and others, *TWIST1* appears to be a key mediator that promotes malignant phenotypes; therefore, *EGFR* signaling utilizes multiple mechanisms to increase *TWIST1* expression, including suppression of its negative regulators (miR-1

and *ETV6*) and activation of its positive regulator (e.g. STAT3) following *EGFR* activation. Therefore, designing novel *TWIST1* inhibitors could sensitize the anti-proliferation effects and reduce the risk of resistance when choosing *EGFR*-TKIs.

Conclusions

Our studies provide a novel and testable hypothesis that connects *ETV6*-*TWIST1* signaling to *EGFR*-TKI resistance during prostate cancer progression. Information about the genetic or expressional statuses of *ETV6* could be valuable for decision making in personalized medicine when considering *EGFR*-based therapeutics. *EGFR* antagonists could achieve better and more-sustainable antitumor responses in combination with *TWIST1*-targeted agents.

Additional file

Additional file 1: Figure Legends and Tables. (ZIP 1350 kb)

Abbreviations

ADT: androgen deprivation therapy; CRPC: castration-resistant prostate cancer; *EGFR*: epidermal growth factor receptor; EMT: epithelial-to-mesenchymal transition; ETS: E26 transformation-specific; *ETV6*: ETS variant gene 6; miR-1: microRNA-1; STAT3: signal transducer and activator of transcription 3; TGFβ: transforming growth factor β; TKI: tyrosine kinase inhibitor; *TWIST1*: Twist family BHLH transcription factor 1

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Availability of data and materials

The human clinical datasets utilized in the study are included in the Methods sections.

Authors' contributions

YCT, TZ, WAK, and YNL conceived the project and designed the experiments. HLY performed the dataset analysis. JJY provided the RasB1 cell line. WYC provided the human samples and performed histomorphometric analysis. All the authors analyzed and interpreted of data. YCT and YNL wrote and all of the authors edited the manuscript.

Ethics approval and consent to participate

We followed the guideline of the Taipei Medical University-Joint Institutional Review Board (approval no.: 201,311,034).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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