Twist Transcriptionally Up-regulates AKT2 in Breast Cancer Cells Leading to Increased Migration, Invasion, and Resistance to Paclitaxel

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Abstract

Metastasis, the cardinal feature of malignant tumors, is an important clinical variable in patient prognosis. To understand the basis for metastasis, we systematically selected for highly invasive cells from breast cancer cell lines, MCF7 and MDA-MB-453, with moderate to low invasive ability using Boyden chamber invasion assay. The four-cycle selected invasive lines, named MCF7-14 and MDA-MB-453-14, respectively, displayed epithelial-mesenchymal transition (EMT) and dramatically enhanced invasive ability. EMT changes were corroborated with decreased level of E-cadherin and increased vimentin, fibronectin, and β3 integrin. Twist, a basic helix-loop-helix transcription factor, and AKT2, a known proto-oncogene, were found to be elevated in the invasive cells compared with the parental. Ectopic expression and knockdown of Twist by short interference RNA resulted in significant increase and reduction, respectively, of AKT2 protein and mRNA expression. Twist bound to E-box elements on AKT2 promoter and enhanced its transcriptional activity. Moreover, silencing AKT2 decreased Twist-promoted migration, invasion, and paclitaxel resistance. Reintroducing AKT2 largely rescued the phenotype resulted from knockdown of Twist in 14 cells, suggesting that AKT2 is a downstream target and functional mediator of Twist. Finally, we observed a 68.8% correlation of elevated Twist and AKT2 expression in late-stage breast cancers as oppose to 13% in early-stage breast cancers. Our study identifies Twist as a positive transcriptional regulator of AKT2 expression, and Twist-AKT2 signaling is involved in promoting invasive ability and survival of breast cancer cells. [Cancer Res 2007;67(5):1979–87]

Introduction

The molecular mechanism of metastasis is still poorly understood. Current models propose a sequential program of metastasis where cancer cells penetrate the basement membrane, intravasate into blood/lymphatic vessels, survive the journey in vasculature, extravasate into secondary sites, and adapt to new host environment (1). Metastatic tumors ultimately lead to poor clinical outcomes; for breast cancer, a significant reduction in the 5-year survival rate from 90% to 20% is observed when comparing localized versus metastasized tumors (2). Metastasis-associated molecular changes include decreased cell-cell junction proteins, such as E-cadherin (3–5), and increased basement membrane degradation proteins, such as matrix metalloproteinases and collagenases (6, 7).

The serine/threonine kinase AKT/protein kinase B, a downstream effector of phosphatidylinositol 3-kinase pathway, has been shown to play a key role in cell survival and growth (8–10). Activated AKT prevents apoptosis via enhancing glucose uptake and utilization, activating nuclear factor-κB pathway, promoting MDM2 nuclear translocation, increasing Bcl-2 or Bcl-xL levels, cytoplasmic sequestering of forkhead-related ligand 1, inactivating Bad and Bax, and inhibiting cytochrome c release from mitochondria (11–13). Additionally, AKT contributes to neoplastic growth via antagonizing p21[cdk1/Waf1] and p27(Kip1), inhibiting glycogen synthase kinase-3β, and promoting mammalian target of rapamycin activity (11). Of the three AKT isoforms, AKT2 has been shown to promote cell motility, invasiveness, and metastasis (14, 15). Moreover, AKT2 is amplified or activated in prostate (9), hepatocellular (16), colon (17), follicular thyroid (18, 19), pancreatic (20–22), ovarian (8, 23, 24), and breast carcinomas (10, 25, 26).

Twist, a basic helix-loop-helix (bHLH) transcription factor, is characterized by a basic DNA binding domain that targets the consensus E-box sequence 5′-CANNTG-3′ and a helix-loop-helix domain that mediates heterodimerization or homodimerization (27). The bHLH protein family has well-described functions in cell growth and differentiation in both vertebrates and invertebrates (28, 29). Developmentally, Twist inhibits myogenic differentiation via interfering with myoD and MEF2 activity (30, 31). Moreover, Twist mediates transcriptional repression by inhibiting two different coactivators, p300 and PCAF (32). Recently, Twist has gained attention as a putative oncogene (33–35), as a contributor to acquired paclitaxel resistance (36), and as a key regulator of metastasis (37). Exogenous overexpression of Twist inhibits apoptosis and promotes colony formation via suppression of the ADP ribosylation factor/MDM2/p53 pathway (33). In the process of metastasis, Twist plays a crucial role by down-regulating E-cadherin and β-catenin, promoting epithelial-mesenchymal transition (EMT), and mediating cell motility and invasiveness (37, 38). Elevated Twist expression leads to higher vascular endothelial growth factor expression, promotes angiogenesis, and correlates with chromosomal instability in breast cancer (39). In addition, increased Twist expression is found in rhabdomyosarcoma (33), melanoma (34), pediatric osteosarcoma (40), T-cell lymphoma (41), gastric (42), prostate (38), and breast carcinoma (37, 43). The elevated Twist expression positively correlates with aggressiveness of cancer and poor survival rate (34, 37, 38).

In an effort to study the molecular mechanism underlying metastasis, we have established a model system using Boyden chamber invasion assay to select highly invasive cells from a metastatic cell line.
population of moderate to low invasive breast cancer lines (MCF7 and MDA-MB-453, respectively). We observed increased Twist and AKT2 expression in the selected highly invasive cells and investigated the possible functional connection between these two proteins. We show that Twist increased mRNA and protein levels of AKT2 in a dosage-dependent manner. In addition, we show that Twist transactivated AKT2 by binding to E-boxes on the AKT2 promoter and drove its expression. Furthermore, blocking AKT2 largely abolished Twist-mediated paclitaxel resistance and invasion activity. Ectopic expression of AKT2 rescued the phenotype of knockdown of Twist. Thus, our results indicate that AKT2 is downstream target of Twist and is a critical player in Twist-promoted metastatic process.

Materials and Methods

Cloning and construction of Twist and related plasmids. Full-length cDNA of Twist was generated by reverse transcription using normal human mammary total RNA as template and followed by nest PCR amplification with the primers derived from human Twist (nest primers: 5'-GCTCTTC-TCCTCTGCCCCG-3' and 5'-CATTAGGTTCTGGGCGCCTG-3'). Green fluorescent protein (GFP)-fused and Myc-tagged Twist were created by digestion of the PCR products with BamHI/EcoRI and by subcloning the PCR products into pEGFP-C2 and pCMV-Tag3B vectors, respectively. The resulted constructs were confirmed by DNA sequencing. AKT2 promoter-driven luciferase reporter plasmids were described previously (44). Deletion mutants of AKT2 promoters were created by PCR.

Antibodies and reagents. Antibodies against E-cadherin, α-catenin, β-catenin, γ-catenin, β1 integrin, and fibronectin were from PharMingen/BD Biosciences (San Jose, CA). Anti-AKT2, anti-Myc, anti-actin, and anti-GFP antibodies were purchased from Cell Signaling (Danvers, MA). Anti-Twist antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Paclitaxel and growth factor–reduced Matrigel were from BD Biosciences (San Jose, CA).

Cell lines, transfection, and tumor specimens. Cells were cultured in DMEM with 10% FCS and antibiotics. Cultures were maintained in a 70% CO2 incubator and fed every 2 days with 10% FCS-DMEM containing 0.4% agar. The cells were then placed into a larger inserts and companion plates were used (Falcon). Cells (2 x 10^5) in a volume of 1.5 mL medium were seeded in the insert and with 2.5 mL of 10% FCS-DMEM as the chemoattractant in the lower chamber. After 24 h of incubation, the invaded cells were recovered by trypsinization and amplified for the next cycle of selection. Statistical analysis was done using two-sample t test, assuming equal variance, and P value was calculated based on two-tailed test.

Soft agar colony formation assay. Cells (1 x 10^5) were suspended in 10% FCS-DMEM containing 0.4% agar. The cells were then placed into a dish containing a hard agar base composed of 10% FCS-DMEM and 0.75% agar. The cultures were returned to the incubator and fed every 2 days with 500 μL of normal growth medium. Photomicrographs of colonies were taken 14 to 21 days later.

Cell survival assay. MCF7 cells stably expressing pCMV-Tag3B or pCMV-Twist were transfected with siAKT2 or siControl. Thirty-six hours after transfection, the cells were treated with or without paclitaxel in ethanol. Thirty-six hours later, percentage cell survival was determined by flow cytometry or trypsin blue staining. Briefly, for flow cytometry, 1 x 10^6 to 2 x 10^6 cells were fixed in 70% ethanol and stained with propidium iodide (PI) solution (50 μg/mL PI and 200 μg/mL RNase A). Cells were resuspended in PBS and analyzed via flow cytometry. For trypsin blue staining, cells were diluted 1:2 with 0.1% trypsin blue in PBS and then counted under a light microscope. Live cells were not permeable to trypsin blue and remained clear and phase bright. Percentage of cell death was calculated via counting cells permeable to trypsin blue. Statistical analysis was done using two-sample t test, assuming equal variance, and P value was calculated based on two-tailed test.

 Luciferase reporter assay. HEK293T or MCF7 cells were seeded in six-well plate and transfected with AKT2-Luc reporter plasmid, pRenilla-luciferase plasmid, and Twist. The amount of DNA in each transfection was kept constant by the addition of empty vector, pCMV-Tag3B. Thirty-six hours post-transfection, firefly and Renilla luciferase were assayed according to the manufacturer’s protocol (Promega, Madison, WI). Luciferase activity was expressed as relative light units. Each experiment was repeated thrice in triplicates.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assay was done essentially as described previously with modifications (45). Briefly, soluble chromatin was prepared from a total of 2 x 10^6 asynchronously growing HEK293T cells that were transfected with Myc-Twist. The chromatin solution was diluted 10-fold with ChIP dilution buffer [1.1% Triton X-100, 1.2 mMol/L EDTA, 167 mMol/L NaCl, 16.7 mMol/L Tris-HCl (pH 8.1), 0.01% SDS, protease inhibitors] and precleared with protein-A beads blocked with 2 μg of sheared salmon sperm DNA and preimmune serum. The precleared chromatin solution was divided and used in immunoprecipitation assays with either an anti-Myc antibody or an anti-HA antibody. Following wash, the antibody-protein-DNA complex was eluted from the beads by resuspending the pellets in 1% SDS and 0.1 mMol/L NaHCO3 at room temperature for 20 min. After reversal cross-link incubation at 67°C, protein and DNA were removed by incubation with 10 μg proteinase K and 10 μg RNase A at 42°C for 3 h. Purified DNA was subjected to PCR with primers specific for four proximal E-box sites within the AKT2 promoter. The sequences of the PCR primers used are as follows: 5'-TGTAAGTGGATCCGTCCTGCTC-3' and 5'-ATCACAGGGGTACAGGACTGGGCTC-3' (E-box 1); 5'-ACCTATTCAACGGAGCATGAC-3' and 5'-TATTATTCCTTCTTATGTTGAGA-3' (E-box 2); 5'-TGCCTCCCAAGCACAGATGATTG-3' and 5'-TCTTTACCTACATGTTGCG-3' (E-box 3); and 5'-TTGATTGCATATTCTGTGGTTGCT-3' and 5'-CTTCTGCTGTCTGGACCTGGG-3' (E-box 4).

Northern and Western blot analysis. Northern blot analysis of total cellular DNA was done according to standard procedures. RNA was extracted using the RNasey purification kits (Qiagen, Valencia, CA). Total RNA was electrophoresed in 1.0% formaldehyde-agarose gels, transferred to Duralon-UVTM membrane (Stratagene, La Jolla, CA), and then hybridized with randomly primed [α-32P]CTP labeled cDNA probes for AKT2. Membranes were exposed to autoradiography and the mRNA levels were visualized and quantified using PhosphorImager analysis (Amersham Biosciences/GE Healthcare, Piscataway, NJ). Western blot analysis was done as described previously (36). Briefly, the cells were lysed with radioluciferase assay buffer [50 mMol/L Tris-HCl (pH 7.4), 150 mMol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mMol/L EDTA, 1 mMol/L phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, 5 μg/mL leupeptin], separated by SDS-PAGE, and immunoblotted with appropriate antibodies as indicated in the figure legends.

Short interference RNA, short hairpin RNA, and retroviral construction. The short interference RNA (siRNA) duplexes were constructed with SilencerTM siRNA Construction kit (Ambion, Austin, TX) following the manufacturer’s instructions. Two siRNAs for human AKT2 (GCAGAGA-TGTGTCTGGCCCTC (siAKT2-2 nucleotides 972–992) and GACAGCG-TTCTCTTCCGCAT (siAKT2-3 nucleotides 1418–1438)) and human Twist
[AAGCTGACGAGATTCAGACC (siTwist1 nucleotides 505–525) and AAGGTACATCGACTTCCTCTC (siTwist4 nucleotides 541–561)] were synthesized. Control siRNA oligonucleotide, which does not match any known human coding cDNA, was designed and used. The siRNA duplexes were reconstituted to 10 μmol/L in sterile RNase-free water. Transfection of siRNA for targeting endogenous genes was done using LipofectAMINE 2000 as per the manufacturer's instructions. The same siRNA targeting regions with a hairpin sequence (TTCAAGAGA) was cloned into pSIREN-RetroQ-linker at BamHI and EcoRI sites (a generous gift from Dr. Domenico Tortorella, Mount Sinai School of Medicine, New York, NY). Sequences are as shown: 5′-gatcgcACAGATGAATTCGCTCTCAAGGAGAGAAGACTGTTCTTGTTTTCGCCG-3′ (shAKT2-2); 5′-gatcgcACAGATGAATTCGCTCTCAAGGAGAGAAGACTGTTCTTGTTTTCGCCG-3′ (shAKT2-3); 5′-gatcgcACAGATGAATTCGCTCTCAAGGAGAGAAGACTGTTCTTGTTTTCGCCG-3′ (shTwist1); and 5′-gatcgcACAGATGAATTCGCTCTCAAGGAGAGAAGACTGTTCTTGTTTTCGCCG-3′ (shTwist4). Retrovirus containing short hairpin RNA (shRNA) was packaged in HEK293T cells and transfected with pVSV-G, pPol-GAG, and respective pSIREN-RetroQ-shRNA. MCF7-I4 was infected with the shRNA virus, selected with puromycin, and used for migration, invasion, and survival assays.

Cell migration and wound healing assay. Migration assay was done using Boyden chamber without coating of Matrigel. Cells were stained and counted. “Wound healing” assay was used to detect the alteration of cell motility. Cells were initially seeded uniformly onto 60-mm culture plates with an artificial “wound” carefully created at 0 h, using a P-200 pipette tip to scratch on the confluent cell monolayer. Microphotographs were taken at 0 and 24 h.

Immunohistochemistry. Immunohistochemistry was done as described previously (39). Anti-Twist and anti-AKT2 antibodies described above were used for the staining. Briefly, paraffin sections of 3-μm thick were cut and probed. After rehydration, samples were treated with solution containing 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. After antigen retrieval in citrate buffer, the sections were incubated with the primary antibody (1:100 in PBS/1% BSA, overnight at 4°C). Then with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA). The signal was amplified by avidin-biotin complex formation and developed with diaminobenzidine followed by counterstaining with hematoxylin, dehydrated in alcohol and xylene, and mounted. The percentage of staining and staining intensity was estimated with ImageJ (NIH, Bethesda, MD).

Results

Invasion selected breast cancer lines assumed a fibroblast-like morphology and displayed EMT-related markers. Using Boyden chamber invasion assay, we intended to select for highly invasive cells from a population of cells with low invasive potential. To find candidate cells for this selection, various breast cancer cell lines were tested for their invasive potential (results not shown). The breast cancer cell lines MCF7 and MDA-MB-453 exhibited relatively low levels of invasion ability were chosen for invasion selection. After four cycles of selection, the selected cells were compared against their respective parental cells for morphology, invasion capacity, and colony-forming ability. The selected cell lines (I4) showed dramatic morphologic change resembling cells undergoing EMT, transitioning from epithelial to mesenchymal-like cells (Fig. 14). We examined protein expression to determine if the I4 cells were indeed different from the parental cells in their surface markers. There was a corresponding decrease in epithelial markers, E-cadherin and cavatin (α, β, and γ), and an increase in mesenchymal markers, vimentin, fibronectin, and β1 integrin, in the MCF7-I4 cells (Fig. 1D). There was a similar increase of fibronectin and β1 integrin; however, there was no change in β-catenin and an increase in γ-catenin in the MB-453-I4 cells. The parental MB-453 cells did not have detectable levels of E-cadherin and α-catenin, which remained the same in the I4 cells. As expected, the I4 cells displayed dramatically increased invasion ability, and MB-453-I4 cells also had increased colony formation ability when compared with their parental cells (Fig. 1B and C). However, parental MCF7 cells have potent colony formation ability to begin with and its invasive selected counterpart did not show a significantly altered colony-forming ability. This observation may be explained by the fact that MCF7 cells lack caspase-3 and is, therefore, more resistant to anoikis-induced apoptosis during anchorage-independent growth (46).

Expression of Twist and AKT2 is elevated in the I4 cells and Twist up-regulates AKT2. The changes in EMT-related protein expression profile and the increased ability for anchorage-independent growth in I4 cells led us to examine known

![Figure 1](https://example.com/figure1.png)
transcription factors involved in EMT process and prosurvival pathways. When compared with the parental lines, I4 cells exhibited an increase in both Twist and AKT2 protein levels (Fig. 2A). Because Twist has been shown to play a pivotal role in EMT (37, 38), we next examined if knockdown of Twist would reverse the phenotype of the I4 cells. MCF7-I4 and MDA-MB-453-I4 were treated with siRNAs targeting two different regions of Twist and control siRNA. Whereas expression of E-cadherin is increased, cell morphology and mesenchymal markers, such as fibronectin, had no changes on knockdown of Twist (Fig. 2B; data not shown). Notably, siRNA-mediated knockdown of Twist in I4 cells resulted in marked decrease of AKT2 protein level (Fig. 2B). This observation led us to further explore the possible relationship between Twist and AKT2 outside of the EMT scheme. Interestingly, ectopic expression of Twist led to a corresponding increase in AKT2 protein level in a dosage-dependent manner (Fig. 2C). Further, Northern blot analysis showed that AKT2 mRNA level was increased on overexpression of Twist in MCF7 cells (Fig. 2D). These data led us to hypothesize that Twist is a positive transcriptional regulator of AKT2.

**Twist transactivates AKT2 promoter.** To determine if Twist directly regulates AKT2 promoter, we examined the promoter and found nine E-box (5′-CANNTG-3′) sequence motifs, representing a series of deletion mutants of the AKT2 promoter (left) were generated and introduced into HEK293T cells together with or without Twist. After 36 h of incubation, cell lysates were prepared for luciferase reporter assay. The experiments were done thrice with triplicate samples for each treatment.
possible binding sites for Twist. Luciferase reporter assay was done to examine if Twist was able to transactivate full-length AKT2 promoter. The result indicated that Twist led to a dosage-dependent increase in AKT2 promoter transactivation as measured by luciferase activity (Fig. 3A).

Twist binds to proximal E-box elements on AKT2 promoter. Serial truncation of AKT2 promoter showed that Twist could transactivate AKT2 promoter with a minimal of one E-box, but maximal transactivation occurred when first four E-boxes were present. The addition of E-boxes 2 and 3 contributed minimally to the transactivation ability of Twist, and the full-length promoter seemed to have repressive elements located between 1.2 and 3.1 kb that inhibited maximal Twist-mediated transactivation (Fig. 3C).

Knockdown of AKT2 decreased Twist-mediated paclitaxel resistance, migration, and invasion. Previous report had shown that Twist conferred paclitaxel resistance in prostate cancer cells (36). We generated MCF7 cells stably expressing Twist and its control line with pCMV-Tag3B vector. When treated with increasing concentration of paclitaxel, we found that MCF7-Twist cells had lower amount of poly(ADP-ribose) polymerase cleavage than that of the MCF7-pCMV cells (Supplementary Fig.S1A). In addition, MCF7-Twist cells also had a lower portion of apoptotic cells compared with that of the MCF7-pCMV control following paclitaxel treatment, suggesting that Twist conferred paclitaxel resistance in breast cancer cells (Supplementary Fig.S1B). When compared with the control, MCF7-Twist cells also had elevated AKT2 level, which was significantly reduced by AKT2 siRNA (Fig. 4A). Knockdown of AKT2 significantly reduced the prosurvival effect of Twist on treatment of MCF7-Twist cells with paclitaxel compared with knockdown of AKT2 in MCF7-pCMV cells (Fig. 4B). These data suggest that AKT2 is responsible for at least in part the Twist-mediated paclitaxel resistance of the cells.

To further show that AKT2 is a downstream target of Twist, we examined if Twist was able to mediate its known migratory and invasive functions in the absence of AKT2. Overexpression of Twist in the parental MCF7 cells led to an increase in migration and invasion ability as measured by Boyden chamber assays (Fig. 4C and D). However, the observed Twist-mediated increase on migration and invasion was significantly reverted when AKT2 was knocked down by siRNA (Fig. 4C and D). These data suggest...
that AKT2 plays an important role in mediating Twist proinvasive functions.

**AKT2 mediates Twist function in MCF7-I4 cells.** To further show the functional link between AKT2 and Twist in controlling cell mobility, invasion, and survival, we asked if AKT2 mediates Twist functions in the selected MCF7-I4 cells, in which endogenous protein levels of Twist and AKT2 were elevated (Fig. 2A and 2B). Parental MCF7 cells, expressing undetectable Twist and low level of AKT2 (Figs. 2A and 2B), were used as controls. Using pSIREN-RetroQ system, AKT2 was stably knocked down by two different shAKT2s targeting different regions of AKT2 in both MCF7 and MCF7-I4 cells (Fig. 5A). As expected, Twist expression was not affected by knockdown of AKT2 (Fig. 5A). In MCF7-I4 cells, reduction in AKT2 resulted in a significant decrease in cell survival, migration, and invasion \( (P < 0.05; \text{Fig. } 5B) \). However, in MCF7 cells, the decrease in AKT2 did not cause a significant decrease in any of the examined functions, although the inhibitory trend was observed \( (P > 0.05; \text{Fig. } 5B) \). These results further indicate that Twist exerts its cellular function in some extent through AKT2.

**Knockdown of Twist in MCF7-I4 can be rescued by reexpression of AKT2.** To provide further functional link between Twist and AKT2, we next examined if AKT2 can rescue the phenotypes resulted from knockdown of Twist in MCF7-I4 cells. We stably knocked down Twist in the MCF7-I4 cells using two different shRNA targeting different regions of Twist. We then reintroduced HA-AKT2 into these cells. Figure 5C shows that Twist level was dramatically reduced by introducing pSIREN-RetroQ-shTwist, further indicating Twist as a transcriptional regulator of AKT2 expression that becomes deregulated during metastatic progression and confirming our initial observations (Fig. 2B). Reintroducing AKT2 into shTwist-MCF7-I4 cells to a level comparable with that of the control significantly rescued the effects of Twist knockdown on cell survival, migration, invasion, and wound healing (Fig. 5D; Supplementary Fig. S2). These data in combination with the findings observed from ectopic expression of Twist and knockdown of AKT2 (Fig. 4) establish the functional link between AKT2 and Twist and indicate that AKT2 is a major target of Twist and mediates Twist function.

**Coexpression of Twist and AKT2 correlates with advanced breast cancer.** Having observed that Twist mediated AKT2 increase in cell culture system, we asked if this regulation is seen in vivo. We examined 12 normal breast and 65 primary breast tumor samples for protein expression of Twist and AKT2 (Fig. 6A). Of the 65 breast tumors, 26 had overexpression of AKT2 and 25 had overexpression of Twist. Of the 25 tumors with elevated Twist, 17 (68%) also had elevated AKT2 levels \( (P < 0.0001) \). Immunohistochemistry of these tumor samples showed that the coexpression of Twist and AKT2 are located specifically to the cancer cells and not to the stroma (Fig. 6B). Furthermore, when grouped by the stage of tumors, we found a striking pattern that the Twist-AKT2 coexpression increased in the late-stage breast tumors. Of the 16 stage III to IV tumors, 11 (69%) tumors had both Twist and AKT2 expression. By contrast, of the 45 stage 0 to II tumors, only 6 (13%) had both Twist and AKT2 expression (Fig. 6C). When grouped by histology, we observed similar elevated trend for coexpression of Twist and AKT2 in the invasive tumor samples versus the noninvasive counterpart (Fig. 6D). Although the coexpression of Twist and AKT2 correlates with advanced breast cancer.

Figure 5. Effects of AKT on Twist function in migration, invasion, and paclitaxel sensitivity in MCF7-I4 cells. A and C, Western blot. Parental MCF7 and/or MCF7-I4 cells were infected with retrovirus containing the indicated shRNAs (A) and subsequently transfected with HA-AKT2 (C). Western blotting analysis was done with the indicated antibodies. B and D, functional assays. The indicated cells were treated with paclitaxel for 36 h. Cell death was assayed by trypan blue staining. Cells were seeded onto the migration/invasion chamber (1 × 10^4 per chamber). After 12 h, chambers were stained and counted for migrated/invaded cells. The experiments were repeated thrice in triplicates samples each. \( P \) values for comparisons are indicated.
Twist and AKT2 between two categories of invasive carcinoma is similar, we observed higher percentage of Twist expression in invasive lobular carcinoma than that of invasive ductal carcinoma. These data suggest that overexpression of Twist and AKT2 correlates with late-stage breast cancer and there is a significant relationship of coexpression of Twist and AKT2, which further support the findings of biochemical and functional links between Twist and AKT2.

**Discussion**

Our study suggests that increased Twist and AKT2 levels correlate with the increased migration/invasion and survival of the selected invasive breast cancer (I4) cells. Whereas knockdown Twist had no significant effect on EMT morphologic changes in the I4 cells, we showed that up-regulation of AKT2 in the I4 cells is resulted from Twist (Figs. 2B and 5C). Twist transcriptionally up-regulates AKT2 and transactivates the promoter by binding to the proximal E-boxes. We also showed that Twist pro-survival and pro-invasive functions are at least in part mediated by AKT2. Further, we observed correlation between elevated Twist and AKT2 in late-stage breast cancers, which suggests the biological relevance of Twist-AKT2 axis in breast cancer progression. Finally, we showed the functional link between Twist and AKT2 by knockdown of AKT2 in both elevated endogenous Twist cells and ectopic expression of Twist clonal cell lines (Figs. 4; 5A and B) as well as by reintroducing AKT2 into Twist knockdown MCF7-I4 cells (Fig. 5C and D). The evidence provided here indicates that AKT2 is transcriptionally regulated by Twist and mediates at least part of Twist functions.

Several lines of observation from previous studies hint at the possible relationship between Twist and AKT2. First, tumors with

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**Figure 6.** Overexpression of AKT2 correlates with elevated Twist and the coelevated AKT2 and Twist associated with late-stage breast cancer. A, representative tumor and normal tissue lysates were analyzed by Western blot with indicated antibodies. Intensity of Twist, AKT2, and actin were quantified via ImageJ software. All the values obtained were normalized to actin. The overexpression of AKT and Twist in tumor samples was scored based on the average values of the normal tissues. B, representative photomicrographs of sections of breast tumor tissue from the same patient stained with polyclonal anti-AKT2 and anti-Twist antibodies. C, overexpressions of AKT2 and/or Twist were observed in late-stage tumors. Percentage of overexpression of AKT2 or Twist (bottom). Right column, the percentage of coexpression of AKT2 and Twist in the different stages of tumor. Approximately 26% of the total patients exhibited overexpression of both AKT2 and Twist. Of the samples that presented with elevated Twist, ~68% had increased AKT2 levels. When samples were stratified according to the stage of the tumor, AKT2 and Twist correlation increases in later stage when compared with the early-stage breast cancer. D, summary of immunohistochemistry analysis. AKT2 and Twist were found to be more frequently detected in invasive breast carcinoma than ductal carcinoma in situ.
acquired paclitaxel resistance displayed Twist gene amplification and AKT2 overexpression/activation (36, 47, 48). Second, both Twist and AKT have been implicated in Wnt signaling pathway (39, 49–51). Wnt pathway inhibitors Frzb/secreted Frizzled-related protein 3 and Wnt inhibitory factor 1 were found to lead to decreased Twist expression and reduced AKT activation (50, 51). Furthermore, both Twist and AKT promotes angiogenesis, cell growth, and survival via altering β-catenin cellular distribution (39). Finally, previous studies have shown that both Twist and AKT2 promote EMT and invasive phenotype of cancer cells (14, 15, 34, 39). These observations suggest that Twist and AKT2 may work in similar pathways in tumor development. Our observations provide direct evidence that AKT2 is a target gene of Twist.

Previous studies have shown that overexpression of AKT1 and AKT2 is a much more frequent event than their gene amplification in human malignancies, suggesting transcriptional regulation of AKT during the tumor development (52). AKT1 has been shown recently to be regulated by signal transducers and activators of transcription 3 (STAT3) and mediate STAT3-induced angiogenesis and cell survival (53, 54). In addition, AKT1 is up-regulated by β-catenin/TCF/LEF and frequent elevated expression levels of AKT1, correlating with enhanced cytoplasmic/nuclear expression of β-catenin, were detected in colorectal carcinoma (55). Whereas AKT2 is up-regulated by MyoD during the muscle differentiation (44), transcriptional regulation of AKT2 has not been shown previously in the cellular processes that are related to carcinogenesis. We have shown in this report that Twist transcriptionally regulates AKT2 and that elevated expression levels of AKT correlate with overexpression of AKT2 in late-stage breast cancer (Figs. 2, 3, and 6). Knockdown AKT2 reduces the effect of Twist on cell migration, invasion, and survival (Figs. 4 and 5).

Our data show that Twist functions as a transcriptional activator of AKT2. Previous studies have shown that Twist functions as a transcriptional repressor and that the action of Twist is regulated by its dimerization with other bHLH-containing transcriptional factors. Post-translational modifications, such as phosphorylation, can alter the dimerization preferences of Twist, either promoting homodimer or heterodimer formation (56). This alteration in Twist dimerization partners ultimately affects the transcription regulatory function of Twist. It has been suggested that Twist as a heterodimer acts as a transcription repressor, whereas Twist homodimer, whose formation is favored by elevated expression, acts as a transcription activator. Recent studies in Drosophila mesoderm development and in human cranial suture patterning suggest that Twist homodimers function as a transcriptional activator (57, 58). In addition, recent report shows Twist as a transcriptional activator of N-cadherin gene in prostate cancer cells (59). The specific mechanism through which dimerization modulates Twist activity on AKT2 promoter remains to be elucidated.

In conclusion, recent evidence suggests that Twist is a major factor participating in tumor development and progression. In human breast cancer, elevated Twist is found in 70% invasive lobular carcinomas (37). Recently, Twist was found to be increased in metastatic lesions of prostate cancer (38). As a novel player in the metastatic program, Twist is gaining rapid attention as a regulator of metastasis. Our finding of the functional link between Twist and AKT2 suggests that targeting Twist and its downstream effectors, such as AKT2, may provide novel therapeutic cocktails for breast cancer intervention.

Acknowledgments

Received 4/25/2006; revised 10/18/2006; accepted 12/29/2006.

Grant support: NIH grants CA29339 and Department of Defense grant DAMD 17-02-0904.

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We thank Dr. Domenico Tortorella (Mt. Sinai School of Medicine, New York, NY) for the pSIREN-RetroQ linker vector and Dr. Jin Q. Cheng (H. Lee Moffitt Cancer Center, Tampa, FL) for his generous gift of AKT2 reporter.

References

Twist Transactivates AKT2


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