Twist Overexpression Induces In vivo Angiogenesis and Correlates with Chromosomal Instability in Breast Cancer

Yelena Mironchik,1 Paul T. Winnard, Jr.,1 Farhad Vesuna,1 Yoshinori Kato,1 Flonne Wildes,1 Arvind P. Pathak,2 Scott Kominsky,2 Dmitri Artemov,2 Zaver Bhujwalla,1 Paul Van Diest,3 Horst Burger,4 Carlotta Glackin,3 and Venu Raman1

Departments of 1Radiology and 2Orthopedic Surgery, Johns Hopkins University, School of Medicine, Baltimore, Maryland; 3Department of Pathology, University Medical Center Utrecht, Utrecht, the Netherlands; 4Institute of Pathology, University of Munster, Munster, Germany; and 5Division of Molecular Medicine, Beckman Research Institute, City of Hope, Duarte, California

Abstract

Aggressive cancer phenotypes are a manifestation of many different genetic alterations that promote rapid proliferation and metastasis. In this study, we show that stable overexpression of Twist in a breast cancer cell line, MCF-7, altered its morphology to a fibroblastic-like phenotype, which exhibited protein markers representative of a mesenchymal transformation. In addition, it was observed that MCF-7/Twist cells had increased vascular endothelial growth factor (VEGF) synthesis when compared with empty vector control cells. The functional changes induced by VEGF in vivo were analyzed by functional magnetic resonance imaging (MRI) of MCF-7/Twist-xenografted tumors. MRI showed that MCF-7/Twist tumors exhibited higher vascular volume and vascular permeability in vivo than the MCF-7/vector control xenografts. Moreover, elevated expression of Twist in breast tumor samples obtained from patients correlated strongly with high-grade invasive carcinomas and with chromosome instability, particularly gains of chromosomes 1 and 7. Taken together, these results show that Twist overexpression in breast cancer cells can induce angiogenesis, correlates with chromosomal instability, and promotes an epithelial-mesenchymal-like transition that is pivotal for the transformation into an aggressive breast cancer phenotype. (Cancer Res 2005; 65(23): 10801-9)

Introduction

It is estimated that >80% of solid tumors are of epithelial origin (1). Such tumors become life threatening once subpopulations of their cellular mass acquire the capability to survive as independent, disorganized, and highly mobile entities that are capable of migrating through the extracellular matrix. They subsequently invade and establish neoplasms within proximal as well as distal tissues (2–4). The cellular transformations that modulate these capabilities seem analogous to some of the cellular changes that are required for normal embryonic development (5, 6). One such necessary developmental trait is the epithelial-mesenchymal transition (EMLT). This process, which begins very early in development of the amniote embryo, produces mesenchymal cells from an epithelial sheet of cells. The resulting cells lose their basal/apical polarity and phenotypic, become elongated, are mobile, and are capable of migrating through the extracellular matrix (7, 8).

The basic helix-loop-helix transcription factor Twist is a major regulator of mesenchymal phenotypes. It is found in mesodermal tissues in humans (9), and in a mouse model system, it has been shown to be required for neural tube closure and is a repressor of myotome differentiation outside of the somites (9). It has been shown that loss of appropriate levels of expression or mutations of normal human Twist result in developmental defects (10–13). Such evidence indicates that Twist expression, a component of mesodermal programming, is necessary for normal vertebrate development. However, it has recently been shown that inappropriate expression of Twist may be oncogenic. Overexpression of Twist in rhabdomyosarcomas inhibited apoptosis and interfered with p53 tumor suppression (14). In addition, increased expression of Twist in four tumor cell lines (nasopharyngeal, bladder, ovarian, and prostate) was found to be associated with resistance to taxol as well as other drugs that similarly disrupt microtubules (15). Moreover, overexpression of Twist has been shown to be a regulator of an epithelial-mesenchymal-like transition (EMLT) in diffuse-type gastric carcinoma (16) and in a mouse mammary tumor cell line (17). Furthermore, MCF-7 cells overexpressing Twist exhibited a deregulated p53 response to γ-radiation, including cell cycle progression and down-regulation of downstream target genes like p21Waf1/Cip1 and MDM-2 (18).

We have developed a human breast cancer cell line (MCF-7) that stably overexpresses human Twist (MCF-7/Twist). In this article, we show that overexpression of Twist produced a transformation of the MCF-7 cell line that exhibited many of the traits representative of an EMLT. In addition, we also report that Twist was able to up-regulate vascular endothelial growth factor (VEGF) synthesis and induce in vivo angiogenesis, characterized by increased vascular volume and vascular permeability as measured by in vivo functional magnetic resonance imaging (MRI). Finally, overexpression of Twist correlates with cytogenetic alterations both in breast tumor samples and in the breast cancer cell line MCF-7/Twist.

Materials and Methods

Immunohistochemistry. Three-micrometer-thick sections were cut from tissue array blocks for immunohistochemistry. Sections were probed with rabbit polyclonal anti-Twist antibody (in house). After rehydration, endogenous peroxidase activity was blocked for 30 minutes in a methanol solution containing 0.3% hydrogen peroxide. After antigen retrieval in citrate C) with the primary antibody (1:100 in PBS/1% bovine serum albumin). The primary antibody was detected using a biotinylated goat anti-rabbit 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 nuclei staining and staining intensity in each core was estimated.

Requests for reprints: Venu Raman, Department of Radiology, Johns Hopkins University School of Medicine, 340 Traway Building, 720 Rutland Avenue, Baltimore, MD 21205. Phone: 410-955-7492; Fax: 410-614-1948; E-mail: vraman2@jhmi.edu.

doi:10.1158/0008-5472.CAN-05-0712

www.aacrjournals.org 10801 Cancer Res 2005; 65: (23). December 1, 2005
Generation of stable MCF-7 clones expressing Twist. MCF-7 breast cancer cells (5 × 10^6) were transfected using LT-1 reagent (Mirus, Madison, WI) with either 1 μg of control vector pcRuz-MycB (Santa Cruz Biotechnology, Santa Cruz, CA) or pcRuz-MycB-Twist. The day after transfection (~12 hours), each plate was split into three 100-mm plates with medium containing 400 μg/mL of G418 (Calbiochem, San Diego, CA). Following transfection, selection was continued for 2 weeks (400 μg/mL of G418), and large healthy colonies were expanded individually into cell lines MCF-7/cont (vector control) and MCF-7/Twist (expressing Twist). The individual cell lines were then verified for Twist expression by immunoblot analyses.

Protein extraction and immunoblot analysis. Total protein from MCF-7/cont and MCF-7/Twist cell lines were extracted using 1X cracking buffer [100 mM/L Tris (pH 6.7), 2% SDS, 12% glycerol] and 1.250 dilution of protease inhibitor (Sigma, St. Louis, MO) and subjected to SDS-PAGE and immunoblot analyses done using antibodies to vimentin (Pharmingen, San Diego, CA), E-cadherin (Transduction Laboratories, Lexington, KY), snail (Santa Cruz Biotechnology), twist (custom made), claudin-7 and claudin-4 (gift of Dr. Scott Kominsky, Johns Hopkins University), and actin (Sigma).

Morphology of MCF-7/Twist cells on Matrigel. Matrigel (300 μL; BD Biosciences, San Jose, CA) was pipetted into each well of a 24-well plate and incubated at 37°C to solidify the Matrigel. Cells (1 × 10^6) in 1 mL of complete medium were then added on top of the solidified Matrigel. The plate was then incubated at 37°C. The medium was replaced as required. Following incubation for 4 days, photographs were taken using a Carl Zeiss inverted microscope fitted with a Nikon Digital Camera. Small interfering RNA (siRNA) knockdown experiments were done as described (18).

Cloning of the claudin-7 promoter reporter construct and reporter assay. The primers 5’-TCCGATGGAGCGCTGATG-3’ (sense) and 5’-TTCCGCTTCAAGAACACT-3’ (antisense) were used to amplify a 1,150-bp human claudin-7 promoter (Cl7-Pr) from WBC genomic DNA. The claudin-7 promoter DNA sequence was verified by sequencing and subsequently cloned into the pGL2-basic vector (Promega, Madison, WI). To fine map the E-box binding sites in the promoter construct, we made four deletion constructs (i.e., Cl7-Pr Δ1 to Δ4).

MCF-7 cells were transiently transfected with increasing amounts of either pcR3.1 empty vector control (Invitrogen, Carlsbad, CA) or pcR3.1-Twist construct (1 μg) along with claudin-7 promoter luciferase construct vector (0.5 μg) and a Renilla luciferase vector (2.5 ng). Transfected cells were assayed using a Dual Luciferase kit (Promega). MCF-7/Twist cells were transfected with claudin-7 promoter luciferase construct (0.5 μg) and Renilla luciferase (2.5 ng) and assayed for luminescence in a luminometer (Berthold Detection Systems, Oak Ridge, TN). The fold change of luciferase units over MCF-7/cont is displayed as a separate histogram for each group of animals. For statistical analysis, median values of PV and PS were determined for each group, and two-tailed t test analysis was used to compare the tumor xenografts of MCF-7/Twist and MCF-7/cont groups.

Comparative genomic hybridization. Comparative genomic hybridization (CGH) was done as previously described (20). Briefly, 300 ng of tumor DNA were labeled with a standard nick-translation reaction with biotin-16-DUTP (Boehringer Mannheim, Indianapolis, IN). Reference DNA (300 ng) from a healthy female donor was labeled with digoxigenin-11-DUTP (Boehringer Mannheim). Labeled DNA fragments were purified from the remaining nucleotides by column chromatography (Qiagen, Valencia, CA). Repetitive sequences were blocked with 40 μg of Cot 1 DNA. The CytoVision 3.1 (Applied Imaging, San Jose, CA) software package was applied for the digital image analysis and subsequent karyotyping.

Tissue array. A tissue array of 144 invasive breast cancer cases was generated with a specialized tissue array precision instrument (Beecher Instruments, Sun Prairie, WI), all characterized by CGH. Each carcinoma was represented by one core. The spot diameter was 0.6 mm, and the distance between spots was 1 mm. Sections of 3-μm thickness were cut for Twist immunohistochemistry.

Results

Twist expression is increased in primary breast carcinomas. Twist expression and its functions have been primarily studied in mesodermal lineages (9, 21–24). In contrast, its expression levels and functions within the epithelial environment have not been extensively studied. As a first step towards evaluating Twist protein expression and its functions in breast epithelium, we did immunohistochemistry on normal breast tissue samples, ductal carcinoma in situ samples, and on primary breast carcinoma samples (grade ≥2, Fig. 1A). Results of the immunohistochemistry data are summarized (Fig. 1B). The results show that high-grade breast tumors express Twist at elevated levels compared with...
EMT is necessary in early embryogenesis, and Twist is a modulator that cancer cells, which undergo an apparent epithelial to mesenchymal transition (EMT), exhibit increased aggressiveness. Upon confirming the expression of Twist in these transformants (Fig. 2C), we observed the three-dimensional growth properties of these cells using Matrigel assays. Although MCF-7/cont cells could not form ductal structures in Matrigel, they retained the ability to grow and spread in a contact-dependent manner (Fig. 2B). In contrast, it can be seen that MCF-7/Twist cells exhibited a growth pattern in Matrigel that resulted in compact spherical structures (Fig. 2B), indicative of a loss of contact inhibition and similar to the growth pattern of the aggressive breast cancer cell line, MDA-MB-231 (26). Thus, overexpression of Twist in MCF-7 cells gives rise to contact-independent growth in Matrigel with the subsequent formation of dense spheroid bodies. These findings, along with the spindle-shaped morphology of MCF-7/Twist cells, are both indicative of an EMT (30). MCF-7/Twist cells have lost E-cadherin and the gain of vimentin are some of the hallmarks of an EMT (30). MCF-7/Twist cells have lost E-cadherin expression while simultaneously up-regulating vimentin (Fig. 2C). In addition, loss of other tight junctions proteins, such as claudin-7 and claudin-4, was also observed in MCF-7/Twist cells. The loss of E-cadherin and the gain of vimentin are two indicators that stable overexpression of Twist in MCF-7 cells induces an EMT. To determine whether or not the observed changes in these protein expression patterns could be a result of Twist activity, we scored for the expression of Snail, a positive modulator of the EMT (31) and a downstream target of Twist (32). Snail was up-regulated in MCF-7/Twist cells providing evidence that Twist’s function as a transcription factor was intact in these cells (Fig. 2C).

To further prove that the observed MCF-7/Twist cell protein marker profile occurred as a result of transcriptional regulation by Twist, we did Affymetrix microarray analyses, with human HGU133 plus 2.0 chips using RNA from MCF-7/cont and MCF-7/Twist cells as probes (Fig. 2D). The increase or decrease of respective transcripts correlated with the protein expression pattern. This result is further evidence that Twist overexpression in MCF-7 cells is responsible for altering the levels of these molecular markers.

**Motility and invasive capabilities of MCF-7/Twist cells.** EMT has been shown to induce motility of cells, an essential component for proper gastrulation (5, 7). Similar phenomenon with respect to motility is also essential for tumor cell invasion (6). To understand this phenotypic trait of EMT, we analyzed the motility and invasive capabilities of MCF-7/Twist cells. In the absence of any mitogenic agent, we observed that 16% of MCF-7/Twist cells were motile compared with 1% of MCF-7/cont cells (Fig. 3A). These results were enhanced in the presence of 10% serum (mitogenic agent), where the percentage of motile MCF-7/Twist cells increased to 45% compared with 14% for MCF-7/cont cells (Fig. 3A). These results indicate that the overexpression of Twist leads to the acquisition of EMT properties, which result in increasing cell motility.

**Figure 1.** Immunohistochemical determination of Twist expression in normal mammary epithelium versus primary breast carcinomas. A, representative photomicrographs of sections of breast tissue stained with Twist-specific, affinity-purified, rabbit polyclonal antibody (custom designed). Sections of normal epithelium show little or no staining, whereas breast tissue samples from ductal carcinoma in situ and invasive ductal carcinoma present a dark staining pattern. B, summary of results from immunohistochemistry on tissue sections from normal breast, ductal carcinomas in situ, and invasive ductal carcinomas obtained using Twist-specific antibody. Staining intensities were categorized as low (+), medium (++), or high (+++) assessed on 80% of the cell population. Abbreviation: NG, no grade.
As tumor invasion requires both motility and invasive capabilities, we examined the invasive potential of MCF-7/Twist cells using Matrigel to simulate the extracellular matrix. As depicted, MCF-7/Twist cells were extremely invasive compared with MCF-7/cont cells (Fig. 3B). More than 45% of the MCF-7/Twist cells were invasive compared with only 1% of MCF-7/cont cells. In addition, the use of siRNA specific for Twist (Fig. 3C) significantly reduced the mobility and invasive capability of MCF-7/Twist cells (Fig. 3A and B). As tumor invasion is necessary for metastasis to occur, it is possible that the Twist overexpression seen in high-grade breast carcinoma samples is essential for the dissemination of tumor cells.

Transcriptional regulation of the claudin-7 gene by twist. As Twist down-regulates claudin-7, we wanted to determine if this down-regulation in MCF-7/Twist cells was a direct consequence of Twist overexpression. Our initial characterization of the claudin-7 promoter sequence enabled us to identify a number of potential E-box sequences that may bind Twist (Fig. 4A). Using a claudin-7 promoter-reporter construct we showed that in MCF-7 cells, Twist in a dose-dependent manner could repress the reporter activity by ~2-fold (Fig. 4B). To map which E-box sites in the claudin-7 promoter were functionally active following binding by Twist, we made deletion constructs (Fig. 4A) and used them in dual-luciferase reporter assays. As seen in Fig. 4C, we found all the four claudin-7 promoter constructs to be down-regulated by Twist by 1.56-fold ($P = 0.0005$), 1.61-fold ($P = 0.0063$), 1.62-fold (not significant), 1.53-fold ($P = 0.03$), and 1.35-fold ($P = 0.0014$), respectively (mean = 1.54-fold). However, we found that the Cl-7 Pr Δ4 construct showed significantly higher activity ($P = 0.0001$) compared with the whole promoter, indicating that this deletion also removed an unidentified repressor binding sequence(s) or unmasked an inducer sequence(s).

Moreover, in MCF-7/Twist cells, the claudin-7 promoter-reporter activity was reduced by a greater degree (~6-fold) when compared with the transient transfection experiments in MCF-7 cells (Fig. 4D). These results indicate that Twist either directly or indirectly causes the transcriptional repression of claudin-7.

Figure 2. Functional analyses of Twist overexpression in breast cells. A-B, altered morphology of MCF-7 cells following Twist expression. MCF-7/cont and MCF-7/Twist cells were grown on plastic and on Matrigel and photographed. C, immunoblotting analysis of EMT markers and scoring for Twist, E-cadherin, vimentin, claudin-4 and claudin-7, snail, and actin loading control, using specific antibodies. D, Affymetrix microarray analysis (HGU133 chips) showing the correlation between protein levels and changes in mRNA levels. The numerical values are averages of two independent experiments and reflect the magnitude of the signal change relative to controls. Arrowheads indicate whether mRNA levels increased (up arrow) or decreased (down arrow).

As tumor invasion requires both motility and invasive capabilities, we examined the invasive potential of MCF-7/Twist cells using Matrigel to simulate the extracellular matrix. As depicted, MCF-7/Twist cells were extremely invasive compared with MCF-7/cont cells (Fig. 3B). More than 45% of the MCF-7/Twist cells were invasive compared with only 1% of MCF-7/cont cells. In addition, the use of siRNA specific for Twist (Fig. 3C) significantly reduced the motility and invasive capability of MCF-7/Twist cells (Fig. 3A and B). As tumor invasion is necessary for metastasis to occur, it is possible that the Twist overexpression seen in high-grade breast carcinoma samples is essential for the dissemination of tumor cells.

Transcriptional regulation of the claudin-7 gene by twist. As Twist down-regulates claudin-7, we wanted to determine if this down-regulation in MCF-7/Twist cells was a direct consequence of Twist overexpression. Our initial characterization of the claudin-7 promoter sequence enabled us to identify a number of potential E-box sequences that may bind Twist (Fig. 4A). Using a claudin-7 promoter-reporter construct we showed that in MCF-7 cells, Twist in a dose-dependent manner could repress the reporter activity by ~2-fold (Fig. 4B). To map which E-box sites in the claudin-7 promoter were functionally active following binding by Twist, we made deletion constructs (Fig. 4A) and used them in dual-luciferase reporter assays. As seen in Fig. 4C, we found all the four claudin-7 promoter constructs to be down-regulated by Twist by 1.56-fold ($P = 0.0005$), 1.61-fold ($P = 0.0063$), 1.62-fold (not significant), 1.53-fold ($P = 0.03$), and 1.35-fold ($P = 0.0014$), respectively (mean = 1.54-fold). However, we found that the Cl-7 Pr Δ4 construct showed significantly higher activity ($P = 0.0001$) compared with the whole promoter, indicating that this deletion also removed an unidentified repressor binding sequence(s) or unmasked an inducer sequence(s).

Moreover, in MCF-7/Twist cells, the claudin-7 promoter-reporter activity was reduced by a greater degree (~6-fold) when compared with the transient transfection experiments in MCF-7 cells (Fig. 4D). These results indicate that Twist either directly or indirectly causes the transcriptional repression of claudin-7.

Figure 3. Increased motility and invasion of MCF-7/Twist cells. A, cell migration assays of MCF-7/cont and MCF-7/Twist cells in the presence and absence of serum. B, cell invasion assays comparing MCF-7/cont and MCF-7/Twist cells. Columns, averages of six independent experiments; bars, SD. $P < 0.001$ (unpaired Student’s t test). C, Western blot analysis of the reduction in Twist protein following transfection with Twist-specific siRNA in MCF-7/Twist cells. Abbreviation: Scr.siRNA, scrambled siRNA.
MCF-7/Twist cells accelerate tumor establishment and growth in the mammary fat pad of severe combined immunodeficient mice. Having established that MCF-7/Twist cells exhibit molecular and phenotypic changes that are characteristic of more aggressive cancer cell lines (26), we next examined whether or not MCF-7/Twist cells could augment tumor growth in SCID mice. To determine tumor growth rate, MCF-7/cont and MCF-7/Twist cells were injected into the mammary fat pad of SCID mice in the presence of estradiol pellets. MCF-7/Twist cells formed tumors within 3 to 4 weeks of inoculation, reaching an average tumor volume of 700 to 800 mm³, whereas at a similar time point, MCF-7/cont cells formed barely palpable tumors (Fig. 5A). It took an additional 5 to 6 weeks for MCF-7/cont cells to reach an average tumor volume of 500 to 600 mm³. These results indicate that Twist overexpression to some extent is able to promote tumorigenesis within our model system.

The rapid growth of MCF-7/Twist xenografts is similar to that of MDA-MB-231, a highly invasive breast cancer cell line (26). H&E staining of the tumor sections clearly show that there is little or no necrosis in MCF-7/Twist xenografts compared with MCF-7/cont xenografts, which exhibited large areas of necrosis (Fig. 5A). It took an additional 5 to 6 weeks for MCF-7/cont cells to reach an average tumor volume of 500 to 600 mm³. These results indicate that Twist overexpression to some extent is able to promote tumorigenesis within our model system.

Vascular volume within the tumor is associated with angiogenic factors secreted by the tumor cells (34). In addition, it has been shown that increased VEGF expression increases microvessel density and is associated with tumor malignancy (35, 36). Therefore, we assayed the levels of VEGF in both MCF-7/Twist and MCF-7/cont xenografts and in mammary epithelial cell lines using ELISA (Fig. 5C and D). Compared with MCF-7/cont cells, MCF-7/Twist cells had 10-fold more soluble VEGF. The amount of VEGF synthesized from MCF-7/Twist cells was more than that produced by MDA-MB-231 (Fig. 5C). Moreover, in tumor xenografts, there was an 4-fold difference between the amounts of VEGF synthesized by MCF-7/Twist compared with MCF-7 (Fig. 5D). This increase in VEGF synthesis could explain, in part, why MCF-7/Twist xenograft tumors showed little or no necrotic areas as well as their rapid growth characteristics.

Elevated vascular flow volume and vascular permeability in xenografts using MCF-7/Twist cells. We did noninvasive in vivo MRI to quantify tumor vascular volume (Vv) and the vascular permeability surface area product (Psv) in the MCF-7/Twist and MCF-7/cont xenograft models. Three-dimensional maps of the Vv and Psv for two typical tumors are shown in Fig. 6A. The body of the animals is outlined by gray shading in the images. The MCF-7/Twist tumor is characterized by a significantly elevated vascular volume (red) and vascular permeability (green) compared with the MCF-7/cont xenografts.
cont tumor. Histograms for tumor $V_V$ and $P_S$ were obtained by pooling all tumor voxels for all MCF-7/Twist and MCF-7/cont tumors (Fig. 6B and C). A greater number of voxels exhibiting high vascular volume and high vascular permeability was detected in the MCF-7/Twist model. Statistical analysis of the data was done using a two-tailed unpaired $t$ test for median values of $V_V$ and $P_S$ for each animal in both groups. The MCF-7/Twist group was found to have a significantly higher $V_V$ ($P < 0.0001$) and higher $P_S$ ($P < 0.002$) than the MCF-7/cont group.

**Overexpression of Twist results in altered $\beta$-catenin levels and localization.** What is the mechanism of increased VEGF synthesis in MCF-7/Twist cells? Given our earlier finding that E-cadherin was down-regulated in MCF-7/Twist cells, we explored the possibility that under these circumstances, $\beta$-catenin expression may not only be mislocalized but may also exhibit aberrant function. $\beta$-Catenin binds to the COOH termini of cadherins at the inner surface of the cytosolic membrane, where it contributes to the molecular linking processes between cell adhesion molecules and the actin cytoskeleton (37). As expected, colocalization of $\beta$-catenin and E-cadherin in MCF-7/cont cells was prominent at the plasma membrane, whereas in MCF-7/Twist cells, these proteins were distributed within the cytoplasm and to a lesser extent within the nucleus (Fig. 6D). In addition, the total amount of $\beta$-catenin and E-cadherin in MCF-7/Twist cells was lower than MCF-7/cont cells (data not shown). The significance of this finding is the redistribution of $\beta$-catenin to the nucleus, where it forms a functional transacting complex by associating with the TCF/LEF-1 transcription factor, enhances the trans-activation of a number of genes, including VEGF and the EMT marker vimentin (38, 39).

**Twist expression in breast tumors correlates with chromosome instability.** Invasive breast tumor samples exhibit significant cytogenetic alterations (20, 40–43) in addition to acquiring EMT phenotypes. In this study, we asked the question whether Twist expression, by itself, could induce genetic alterations. The comparison of 144 breast tumor samples with CGH data indicates that tumors that expressed Twist also have at least 2-fold more genetic alterations than tumors without Twist expression (Table 1). Our results indicate that invasive lobular as well as invasive ductal carcinomas show an up-regulation in Twist expression in 63% and 75% of the cases studied, respectively ($n = 144$). An earlier report using microarray data of a smaller sample size ($n = 57$) shows a much lower expression in invasive ductal carcinomas (17).

**Discussion**

Tumor progression and invasion is a complex biological process that involves remodeling of stromal tissue by invading cells (3, 4). This is similar to the formation of the mesoderm before gastrulation during embryogenesis (5–7). Our results indicate that some of the dormant developmental pathways are activated during invasion of tumor cells by the expression of the Twist gene. These results show that Twist expression is more pronounced in invasive breast carcinomas compared with the normal breast tissue, indicating its possible involvement in tumor progression.

![Figure 5](image_url)
Overexpression of Twist in MCF-7 cells generated an EMLT as manifested by the acquisition of increased motility and invasion. As the EMT in embryogenesis is characterized by the rapid movement of cells, it is likely that molecular pathways associated with Twist activation are involved in tumor invasion. Affymetrix microarray analyses along with protein expression data of MCF-7/Twist cells showed the activation of Snail, a known repressor of E-cadherin (31) in conjunction with loss of E-cadherin expression. As loss of tight or adherens junctions proteins are essential for EMT (3, 5–7), our results are consistent with the observed phenotype of MCF-7/Twist cells. E-cadherin, a transmembrane glycoprotein that mediates calcium-dependent intercellular adhesion, is involved in epithelial cell-to-cell adhesion and is an important regulator of morphogenesis (8). Loss of E-cadherin expression has been associated with the acquisition of invasiveness in many advanced tumor types (8). As E-cadherin is a marker of epithelial cells, loss of E-cadherin indicates the loss of the epithelial phenotype. There is evidence indicating that the germ line mutation of E-cadherin predisposes individuals to diffuse gastric and breast cancer (44–48). In addition to loss of E-cadherin, other EMT markers, such as vimentin, were also induced in MCF-7/Twist cells. Vimentin, which is an intermediate filament protein, is specifically regulated during embryonic development and cellular differentiation of mesenchymal cells (39). Altered vimentin expression is associated with the ability of tumor cells to invade adjacent tissues and migrate into the body (26, 39). Evidence from some reports indicates that vimentin may establish a link between the extracellular matrix and the nucleus thus modulating cellular functions (39). The loss of E-cadherin and the gain of vimentin indicate that overexpression of Twist in MCF-7 cells induces an EMLT thus facilitating increased motility and invasiveness.

Figure 6. MRIs of MCF-7/cont and MCF-7/Twist xenografts in SCID mice. A, representative three-dimensional volume rendering of vascular volume (red) and vascular permeability surface area product (green) of the tumors obtained with in vivo dynamic MRI. B, histogram of the vascular volume in MCF-7/cont- and MCF-7/Twist-xenografted tumors (pooled data for five animals in each group). C, histogram of the vascular permeability in MCF-7/cont- and MCF-7/Twist-xenografted tumors (pooled data for five animals in each group). D, altered localization of β-catenin and E-cadherin in MCF-7/Twist cells. MCF-7/cont and MCF-7/Twist cells were scored for β-catenin (left) and E-cadherin (middle) expression using specific antibodies. Right, merged photos of β-catenin and E-cadherin expression in the two cell lines.
A question that arises is whether or not the procurement of EMLT is sufficient to augment tumorigenesis? Based on our results, MCF-7/Twist cells were able to establish a tumor within 3 to 4 weeks of orthotopic implantation in the mammary fat pad of SCID mice compared with 8 to 9 weeks for MCF-7/cont cells. This indicates that Twist expression, besides inducing an EMLT, can also promote tumor growth. Furthermore, the tumor architecture of the MCF-7/Twist xenograft tumors exhibited little or no necrosis compared with MCF-7/cont xenografts of similar volume, indicating a well-developed vasculature, which should facilitate tumor growth.

Establishing a vascular system within the tumor environment requires angiogenic factors, such as VEGF (34). The MCF-7/Twist xenografts had a 4-fold increase of VEGF when compared with MCF-7/cont xenografts. Similar results were also obtained in vivo using MCF-7/Twist cells. This could be due to the loss of E-cadherin and the mislocalization of β-catenin to the nucleus. Previous studies have shown that the catenin-cadherin complex is required to maintain the mammary gland architecture and influences polarity, cell fate, and motility of epithelial cells (49). Perturbation of the β-catenin/E-cadherin complex can result in the nuclear localization of β-catenin, which is associated with increased vimentin and VEGF expression along with a potentially more invasive phenotype (50). Overexpression of Twist in MCF-7 cells did increase vimentin expression. Taken together, the results from our group and others support the possibility that perturbed expression of β-catenin can induce vimentin, among other genes, which in turn induces an EMLT and the acquisition of increased motility and invasive potential.

To further understand the role of Twist in breast cancer tumorigenesis, we did functional MRI on MCF-7/Twist and MCF-7/cont xenograft tumors. The data obtained for MCF-7/Twist xenografts indicate that not only do they synthesize more VEGF, but they also seem angiogenically more active. The MRI data shows elevated vascular permeability surface area and vascular volume in MCF-7/Twist-derived tumors compared with MCF-7/cont strongly supporting the observation that Twist overexpression in vivo results in elevated expression of functional VEGF. These MRI data are consistent with the role of VEGF as a potent angiogenic and permeability factor.

The development of a highly invasive breast cancer phenotype requires the coordination of many different molecular changes, which are a consequence of genomic alterations. We found an association between increased cytogenetic alterations and Twist overexpression in breast tumors. Chromosomes 1, 7, 15, and 17 were amplified in human breast tumors expressing Twist. Taken together with the in vivo MRI data, our results show that Twist overexpression induces an EMLT, promotes angiogenesis, and correlates with chromosome instability in breast cancer and could be considered as a possible therapeutic target for preventing invasion and metastasis.

Acknowledgments
Received 3/2/2005; revised 8/29/2005; accepted 9/14/2005.
Grant support: NIH grant 1R01CA097226 (V. Raman).
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 1744 solely to indicate this fact.
We thank Petra van der Groep for doing the Twist immunohistochemistry.

Table 1. Twist overexpression correlates with chromosomal instability in breast cancer

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Samples</th>
<th>Twist signal</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive ductal carcinoma</td>
<td>93</td>
<td>70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive lobular carcinoma</td>
<td>27</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medullary, mucinous, tubular</td>
<td>24</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>and cribriform carcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive ductal carcinoma</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive lobular carcinoma</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medullary, mucinous, tubular</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>and cribriform carcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

No. breast tumor samples analyzed by CGH

<table>
<thead>
<tr>
<th>% Tumors expressing Twist</th>
<th></th>
<th>Chromosome amplification in Twist expressors</th>
</tr>
</thead>
<tbody>
<tr>
<td>144</td>
<td>70</td>
<td>1, 7, 15, and 17</td>
</tr>
</tbody>
</table>

NOTE: Correlation between Twist expression in breast cancer and cytogenetic alterations in 144 breast tumor samples with comparative genomic hybridization data.

Abbreviation: CGH, comparative genomic hybridization.

References
Twist Expression Promotes Tumor Growth

Yousfi M, Lasmoles F, El Ghouzzi V, Marie PJ. Twist 12.
Maestro R, Dei Tos AP, Hamamori Y, et al. Twist is a potential oncogene that inhibits apoptosis.

32. Leptin M, Twist and snail as positive and negative regulators during Drosophila mesoderm development. genetics of epithelial-mesenchymal transition in breast cancer.
Twist Overexpression Induces \textit{In vivo} Angiogenesis and Correlates with Chromosomal Instability in Breast Cancer

Yelena Mironchik, Paul T. Winnard, Jr., Farhad Vesuna, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/23/10801

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2005/11/28/65.23.10801.DC1

Cited articles
This article cites 47 articles, 11 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/23/10801.full#ref-list-1

Citing articles
This article has been cited by 30 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/23/10801.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, use this link http://cancerres.aacrjournals.org/content/65/23/10801.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.