α-Tubulin Acetylation Elevated in Metastatic and Basal-like Breast Cancer Cells Promotes Microtentacle Formation, Adhesion, and Invasive Migration

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Abstract

Metastatic cases of breast cancer pose the primary challenge in clinical management of this disease, demanding the identification of effective therapeutic strategies that remain wanting. In this study, we report that elevated levels of α-tubulin acetylation are a sufficient cause of metastatic potential in breast cancer. In suspended cell culture conditions, metastatic breast cancer cells exhibited high α-tubulin acetylation levels that extended along microtentacle (McTN) protrusions. Mutation of the acetylation site on α-tubulin and enzymatic modulation of this posttranslational modification exerted a significant impact on McTN frequency and the reattachment of suspended tumor cells. Reducing α-tubulin acetylation significantly inhibited migration but did not affect proliferation. In an analysis of more than 140 matched primary and metastatic tumors from patients, we found that acetylation was maintained and in many cases increased in lymph node metastases compared with primary tumors. Proteomic analysis of an independent cohort of more than 390 patient specimens further documented the relationship between increased α-tubulin acetylation and the aggressive behaviors of basal-like breast cancers, with a trend toward increased risk of disease progression and death in patients with high-intensity α-tubulin acetylation in primary tumors. Taken together, our results identify a tight correlation between acetylated α-tubulin levels and aggressive metastatic behavior in breast cancer, with potential implications for the definition of a simple prognostic biomarker in patients with breast cancer. Cancer Res; 75(1); 203–15. ©2014 AACR.

Introduction

Advances in detection and treatment have greatly increased survival rates of patients diagnosed with localized or regional breast cancer. However, for patients diagnosed with metastatic breast cancer, survival rates drop dramatically (1). Current treatments are largely successful at prolonging patient survival, but they are not sufficient to prevent metastasis (2). Because most breast cancer–related deaths are due to secondary disease, finding targets to treat or prevent metastasis is an important therapeutic priority.

In order for a primary tumor to metastasize, cancer cells must leave the breast and enter the blood or lymphatic system. Once detached, these circulating tumor cells (CTC) attach and/or arrest at secondary sites before extravasation and metastatic outgrowth. The cytoskeleton, composed of actin microfilaments, microtubules, and intermediate filaments, plays a vital role in metastatic dissemination (3). Tumor cell reattachment has been shown to be dependent on stable microtubules in vivo (4–8), while the stability of the microtubule network has also been implicated in the control of migration (9, 10), highlighting a potential therapeutic target for both attached and suspended disseminated cells.

Breast cancer cells produce long and dynamic microtubule-based membrane protrusions, termed microtentacles (McTN), upon detachment (8, 11–13). Importantly, invasive breast tumor cells produce significantly higher frequencies of McTNs compared with noninvasive cell lines (14). These protrusions encircle adjacent cells to promote cell–cell aggregation and facilitate reattachment of tumor cells to an extracellular matrix, endothelial monolayer, and retention in the lungs of mice (7, 13, 14). McTNs can be enhanced by actin depolymerization but are dependent upon microtubule stability (12, 13). Data support a model in which McTNs are generated when the physical force generated by outwardly expanding microtubules overcomes the contractile force of the actin cortex underlying the plasma membrane (8). Inhibition of McTNs by microtubule-destabilizing drugs significantly

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reduces cell–cell and cell–substrate reattachment efficiency of breast tumor cells (11). Conversely, increased microtubule stability enhances reattachment for in vitro and in vivo metastasis models (7, 15).

Posttranslational modifications (PTM) of α-tubulin can control diverse microtubule functions, such as signaling, trafficking, and cellular tensegrity (16, 17), but we are only beginning to uncover many functions that could affect cancer progression and metastasis. Acetylation of α-tubulin, a well-known marker of stabilized microtubules, occurs on lysine 40 (K40) by the α-tubulin acetyltransferase 1 (αTAT1; refs. 16, 18, 19) and can be reversed by histone deacetylase 6 (HDAC6) and sirtuin 2 (SIRT2; ref. 20). Studies suggest high HDAC6 levels and low acetylated α-tubulin are associated with good prognosis and increased disease-free survival of patients with breast cancer (21, 22), but the mechanisms behind this correlation and the role of this PTM in metastatic breast cancer are not clear.

Detyrosination is the only α-tubulin PTM associated with microtubule stability that has been found to play a significant role in McTN formation and reattachment of suspended breast tumor cells (11). However, previous studies could not establish a correlative trend between cancer invasiveness and detyrosination of α-tubulin (14). Because CTC reattachment is dependent upon stable microtubules in vivo (5), an alternative α-tubulin PTM associated with microtubule stability was investigated. In this study, we present a novel role for α-tubulin acetylation in breast cancer. We find a significant association between metastatic breast cancer cell lines and high acetylation of α-tubulin that extends along the length of McTN protrusions. Mutation of the specific lysine 40 acetylation site on α-tubulin as well as enzymatic modulation of this PTM has a significant impact on McTN frequency and cancer cell reattachment. Investigation into chemotaxis of attached breast tumor cells finds acetylated α-tubulin is also necessary for migration. Furthermore, matched primary and metastatic tumor arrays containing tissue from more than 140 patients show acetylation is maintained and increased in many nodal metastases while large-scale proteomic studies of more than 390 patients link this modification to the aggressive basal-like subtype. There is also a trend of increased risk of disease progression and death when α-tubulin acetylation is high in a patient’s primary tumor. Acetylation of α-tubulin may promote a more metastatic phenotype through its effects on reattachment and migration while serving as a marker for basal-like breast cancer and a potential prognostic indicator.

Materials and Methods

Cell culture

MCF-7, BT-20, BT-549, and Hs578T cells were obtained from the American Type Culture Collection. MDA-MB-231 cells were obtained from Addgene. AcGFP1-C1 was obtained from Clontech. Transient transfections used Fermen-
tas ExGen 500 in vitro transfection reagent (Thermo Scientific) according to the manufacturer’s protocol. Stable cell lines were selected 3 days after transfection. Stable pooled clones were verified after antibiotic selection by GFP expression and immunoblot.

Immunoblot

Cells were harvested as previously described (14). Protein (20 μg) was separated by SDS-PAGE on 4% to 12% NuPage MES Bis-Tris gels (Life Technologies). Membranes were blocked with 5% milk/TBST for 1 hour at room temperature before overnight incubation at 4°C with primary antibody: acetyl-α-tubulin (Lys40; D20G3) XP rabbit mAb (1:1,000; Cell Signaling Technology), anti-detyrosinated α-tubulin (1:1,000; Abcam), monoclonal anti-α-tubulin Clone DM1A (1:5,000; Sigma-Aldrich), and GFP (1:5,000; Santa Cruz Biotechnology) in 5% milk/TBST. Densitometry was calculated using three independent immunoblots with ImageJ (NIH, Bethesda, MD).

Attached and suspended immunofluorescence

Cells were suspended for 30 minutes in ultra-low-attach plates (Corning) then spun down onto poly-L-lysine–coated coverslips. Cells were fixed with 3.7% formaldehyde/PBS, washed, permeabilized in 0.25% Triton-X 100/PBS, blocked in 5% BSA/0.4% PBS, and incubated overnight at 4°C in 2.5% BSA/0.4% PBS with primary antibody. Secondary antibody was added 1:500 in PBS with Hoechst. Images were acquired using an Olympus FV1000 confocal microscope (Olympus) and analyzed with ImageJ.

Live cell imaging and McTN scoring

Live cell imaging and McTN scoring was performed as previously described (7). Briefly, cells were stained with CellMask Orange plasma membrane stain (Life Technologies) and sus-
pended for 30 minutes in ultra-low-attach plates (Corning). Cells were blindly scored for McTNs using an Olympus CKX41 inverted fluorescent microscope. Images were taken using the MicroSuite Five software (Olympus). Transiently transfected cells were con-
firmed to be GFP-positive before counting.

Cell proliferation assay

The number of viable cells in proliferation was determined by the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer’s protocol. Briefly, 10,000 cells per well were plated in triplicate/time point in a 96-well plate in full-serum media. CellTiter was added at $t = 0$ and every 24 hours thereafter for 120 hours; absorbance was read at 490 nm. Viability in serum-free conditions was monitored over the same time, except $t = 0$ was marked by the washout of full-
serum media and the addition of serum-free media. Average absorbance at each time point was divided by the average absorbance reading at day 0 to account for differences in cell number at plating.

Cell reattachment and migration assays

Real-time cell reattachment and chemotaxis was measured using the xCELLigence RTCA DP device (ACEA Biosciences, Inc.) according to the manufacturer’s protocol. Briefly, 20,000 cells per well were added to electrode-containing microtiter plates (E-plate 16) for reattachment. MDA-MB-231/MCF-7 (40,000 cells/well).
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and BT-549 (20,000 cells/well) were added to each CIM-plate 16 for migration. CIM-plates are similar to a Boyden chamber, containing an upper and a lower chamber separated by a poly-ethylene terphthalate membrane with 8-µm pore size. The membrane has gold electrodes on the bottom of the upper chamber to detect cells moving into the lower chamber. The chemoattractant used was 5% FBS. Reattachment and migration rates were quantitatively recorded as Cell Index (a change in electrical impedance of the current flowing through the electrodes). Impedance was recorded every 1 to 5 minutes for 24 hours for reattachment and every 15 minutes for 24 hours for chemotaxis. Raw data were exported to Microsoft Excel. Graphs shown are representative of a single experimental run ± standard deviation of 3 wells. The underside of the CIM-plate upper chamber was stained with Cell Stain (Millipore) to visualize migrated cells at 24 hours with a Nikon SMZ1500 stereomicroscope attached to a Nikon digital camera DXM1200 using ACT-1 Software Version 2.62 (Nikon).

Patient samples
Breast cancer and matched metastatic carcinoma of lymph node tissue arrays BR1005a, BR10010a, and BR1001 were obtained from US BioMax, Inc. Samples from 144 patients were stained by the University of Maryland Greenebaum Cancer Center (UMGCC; Baltimore, MD) Pathology Biorepository and Research Core using the anti-acetylated tubulin antibody. Blind scoring of samples was carried out by Dr. Olga B. Ioffe, head clinical pathologist for breast and gynecologic cancers. Images were scanned using the Aperio System and captured using ImageScope Viewer (www.aperio.com). Supplementary Fig. S4 contains patient ages and histologies.

Reverse phase protein array
Breast cancer patient primary tumors (412) from The Cancer Genome Atlas (TCGA) Breast Invasive Carcinoma RPPA Set 041011-0035 were assayed for acetylated α-tubulin intensity as previously described (23). Briefly, proteins extracted from patient tumors were printed on slides, probed with anti-acetylated tubulin, and a signal was obtained and visualized by colorimetric reaction. Scanned slides were analyzed using Microvigen software (VigenTech Inc.). Dilution curves were fitted with a logistic model developed by MD Anderson Cancer Center (Houston, TX) and concentrations were normalized to correct for loading. Read-out of loading-corrected intensity was calculated as "Normalized Linear Value." Data from 10 patients were excluded because molecular subtype (classified via PAM50; ref. 24) was not determined. Data from 5 male patients and 5 patients subtyped as "normal" were also excluded. Patients (392) were matched with clinical information (current as of July 2014) presented by the TCGA Research Network (TCGA Data Portal: https://tcga-data.nci.nih.gov/tcga/). Acetylation intensity was classified as high versus low if it was above or below the median acetylation intensity, respectively, for n = 392 patients. Supplementary Fig. S5 contains clinical information.

Survival analysis
The study population for survival analysis included 277 patients analyzed through reverse phase protein array (RPPA). Exclusion criteria included prior treatment or neoadjuvant chemotherapy, unknown nodal status, and follow-up time of less than 1 year (<365 days). Patients diagnosed before 1998 were also excluded from survival analysis due to FDA approval of trastuzumab and letrozole that year (25, 26). Overall survival (OS) was time from date of diagnosis to date of death from any cause, censored at date of last contact. Progression-free survival (PFS) was time from date of diagnosis to the earlier of disease recurrence or death from any cause. Patients alive without recurrence were censored at date of last contact. Supplementary Table S1 contains clinical information.

Statistical analysis
The McNemar test was used to test the equality of binary acetylation rates from two populations (primary tumor and metastases) with the data that are paired and dependent, because tumor and metastases rates are obtained from the same patients. The Welch two-sample test was applied to determine significance of basal-like acetylation intensity versus non-basal subtypes. Significance testing of densitometry and McTN scoring was assessed using t test.

Survival analysis was conducted by Dr. Olga G. Goloubeva, faculty member of the Biostatistics and Bioinformatics Shared Service of UMGCC. The Kaplan–Meier approach was used to estimate OS and PFS functions. Hazard ratios (HR) with the corresponding 95% confidence intervals (CI) were estimated using the Cox regression model to assess plausible association between tubulin acetylation and patients' OS as well as PFS. The proportionality assumption for hazards was tested using martingale and Schoenfeld residuals. Acetylation intensity was log-transformed to smooth the distribution and decrease variability. Patients were grouped according to acetylation intensity using the 25th, 50th, and 75th quartiles of the marker's distribution. OS and PFS were estimated for the predefined patient groups and compared by the log-rank test. Event times were truncated at 8.5 year. Statistical analysis was conducted in R (3.0.3, x64) and SAS (v.9.3; SAS Institute Inc.).

Results
Acetylation of α-tubulin is significantly increased in metastatic breast cancer cells, correlates with increased McTN frequency, and is enriched in McTNs

Given previous data showing that stable microtubules are associated with increased CTC reattachment in vivo (5), we sought to determine whether α-tubulin PTDs associated with microtubule stability are differentially represented in breast cancer cells. Acetylation and detyrosination of α-tubulin were investigated in nonmetastatic (MCF-7, MDA-MB-453, and BT-20) and metastatic (MDA-MB-231, BT-549, and Hs578T) cell lines (27). Only acetylation of α-tubulin was significantly associated with the metastatic cell lines, whereas detyrosination was not (Fig. 1A and B and Supplementary Fig. S1A). There is over a 3-fold difference between the average acetylation in nonmetastatic cell lines compared with metastatic cell lines (Fig. 1B).

Immunofluorescence was performed to visualize differences in localization and structure of acetylated α-tubulin, as compared with total α-tubulin in the cell panel. The nonmetastatic cell lines MCF-7 and MDA-MB-453 exhibit low basal α-tubulin acetylation, with only BT-20 showing minimal acetylation (Fig. 1C). In contrast, metastatic MDA-MB-231, BT-549, and Hs578T cell lines display robust acetylation of α-tubulin with bundling or increased density of acetylated microtubules radiating from the perinuclear region.
Upon suspension, breast tumor cells produce McTNs, membrane-based protrusions dependent upon microtubule stability that can aid in suspended cell reattachment (7, 11–14). Given the increased tubulin acetylation detected in more metastatic cell lines that remains elevated under suspended conditions (Supplementary Fig. S1B), blinded quantitation of McTN frequencies was carried out. The metastatic cell lines with higher acetylation of α-tubulin have significantly more McTNs than the nonmetastatic lines (Fig. 1D). Figure 1E shows representative images of nonmetastatic and metastatic suspended cells in which the metastatic cells produce increased McTNs. The remainder of the suspended cell panel is shown in Supplementary Fig. S1C.

Detyrosination is the only α-tubulin PTM to date that has been reported to be enriched in McTNs; however, detyrosination does not directly correlate with increased McTN occurrence or invasiveness (11). Given that increased acetylation correlated with higher McTN frequency in metastatic lines, the role of α-tubulin acetylation in McTN formation was examined. Immunofluorescence of suspended metastatic cell lines shows acetylated α-tubulin extends along the lengths of McTNs (Fig. 1F, arrows), highlighting that this PTM is a constituent of McTNs.

K40R α-tubulin mutation decreases endogenous acetylation and McTN frequency

The increase in acetylation observed in metastatic cell lines and enrichment of this PTM in McTNs propelled further investigation into the mechanistic role of α-tubulin acetylation in McTN formation. We used a point mutation on α-tubulin to determine whether decreasing α-tubulin acetylation could affect McTNs. Acetylation occurs on lysine 40 of α-tubulin (K40), a highly conserved site that affects microtubule stability (28, 29). Previous research has shown the lysine 40 to arginine α-tubulin point mutation decreases endogenous acetylation and McTN frequency.
mutation (K40R) is acetylation-resistant but can still incorporate into the microtubule polymer (30).

MDA-MB-231 and BT-549 cells were selected to investigate the effects of the K40R mutation given their high endogenous acetylation and McTN frequency. Immunofluorescence was carried out on transient transfections with either K40R α-tubulin-GFP or a control α-tubulin-GFP to compare the effects of this mutant on acetylation of the α-tubulin network. MDA-MB-231 and BT-549 cells expressing the K40R α-tubulin-GFP showed major disruption of acetylated α-tubulin filaments after 24 hours (Fig. 2A, top row, arrows), compared with adjacent untransfected cells (Fig. 2A, top row, arrowheads) or transfection of the α-tubulin-GFP control (Fig. 2A, bottom row). Under attached and suspended conditions, MDA-MB-231 and BT-549 cells stably expressing the K40R α-tubulin-GFP mutant showed decreased endogenous acetylation of α-tubulin, as compared with α-tubulin-GFP control cells (Fig. 2B). We found no significant difference in proliferation between cells stably expressing K40R α-tubulin-GFP and the α-tubulin-GFP control (Supplementary Fig. S2A and S2B).

Because K40R expression decreased endogenous acetylation and significantly reduced acetylated microtubules, the impact of reducing α-tubulin acetylation on McTN formation in cell lines with high McTN frequency was investigated. MDA-MB-231 and BT-549 control cells exhibit numerous long McTNs when suspended (Fig. 2C, arrows). However, when these metastatic cells stably express the nonacetylatable K40R α-tubulin mutant, the McTN protrusions are significantly reduced (Fig. 2C, top, and D). McTN frequency was reduced by more than 45% in MDA-MB-231 and 62% in BT-549 K40R expressing cells, as compared with controls (Fig. 2D). Additional McTN counts were carried out on transiently transfected MDA-MB-231 and BT-549 cells to ensure that the process of creating the stable cell lines did not adversely affect McTNs. Similar to what was seen in the stable lines, both metastatic cell lines significantly decreased McTN frequency when...
transiently expressing the K40R mutant (Supplementary Fig. S2C).

**Overexpression of αTAT1 increases tubulin acetylation and enhances McTN frequency**

Because reducing acetylation of α-tubulin decreased McTN formation and frequency in highly acetylated metastatic breast tumor cells, we reversed this molecular mechanism to determine whether increasing acetylation in a nonmetastatic cell line would promote McTNs. The αTAT1 was recently demonstrated to specifically acetylate α-tubulin on lysine 40 (18, 19). MCF-7 cells were selected to overexpress αTAT1 because they have low endogenous acetylation and McTN frequency.

Overexpression of αTAT1-GFP (19) in MCF-7 cells caused robust acetylation of α-tubulin throughout the cytoplasm of transfected cells (Fig. 3A, top row, arrows), whereas the GFP control (Fig. 3A, bottom row, arrows) and nontransfected cells (arrowheads) were unaffected. Overexpression of αTAT1 or the GFP control did not affect the overall α-tubulin network (Supplementary Fig. S3A). Concurrently, immunoblot shows that αTAT1 overexpression greatly increased endogenous acetylation of α-tubulin in both attached and suspended MCF-7 cells, as compared with the GFP-control (Fig. 3B). Transient overexpression of αTAT1-GFP or the GFP control did not significantly affect proliferation (Supplementary Fig. S3B).

αTAT1-GFP or the GFP control MCF-7 cells were then suspended to determine the effects of increased acetylation on McTN formation and function. We found that overexpression of αTAT1 significantly increased McTN frequency by approximately 2-fold over control (Fig. 3C). Because of the significant difference in McTN frequency, we then examined if αTAT1-induced acetylation of α-tubulin localized along the lengths of McTNs. Suspended cell immunofluorescence revealed that acetylated α-tubulin extended within McTNs in the αTAT1-overexpressing cells (Fig. 3D, top row, arrows) but not in the GFP-transfected controls (Fig. 3D, bottom row).

**Altering acetylation of α-tubulin influences the reattachment of suspended breast tumor cells**

McTN function is evaluated by the ability of detached cells to reattach to model one of the early steps for CTC retention in distant tissues (7). To assess how the reduction in acetylation affects reattachment of metastatic cell lines, MDA-MB-231 and BT-549 cells expressing the K40R stable mutation were analyzed. The K40R-mutant α-tubulin–expressing cells reattached at a significantly decreased rate over 2 hours, as compared with controls (Fig. 4A and B). It is interesting to note that BT-549 cells had a greater difference in McTN frequency between those stably expressing the K40R mutant and the α-tubulin control (Fig. 2D). This could explain the larger difference in reattachment rates, as compared with the significant but less drastic reduction in reattachment in the MDA-MB-231 cells.

In complementary experiments, increasing acetylation by overexpressing αTAT1 in low acetylated and nonmetastatic MCF-7 cells revealed that increased acetylation significantly increases reattachment, compared with the GFP control (Fig. 4C). This elevation in attachment efficiency parallels the increased McTN counts in Fig. 3C, not only confirming McTN function but demonstrating for the first time the importance of acetylation in cell reattachment.

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**Figure 3.** αTAT1 significantly increases α-tubulin acetylation and McTNs.

A, MCF-7 cells were transiently transfected with αTAT1-GFP or GFP control and were subjected to immunofluorescence for acetylated α-tubulin (red). Transfected cells are indicated by arrows; nontransfected cells are indicated by arrowheads. DNA is stained in blue. Scale bar, 20 μm. B, immunoblot of cells lysed after 24 hours transfection under attached or 30 minutes suspended conditions. C, McTN counts of MCF-7 cells transiently transfected with αTAT1-GFP or GFP control. Data represent n = 3 in triplicate ± standard deviation. **, P < 0.01. D, cells were subjected to suspended cell immunofluorescence for acetylated α-tubulin (red). Arrows, McTNs. Scale bar, 10 μm.
Decreasing α-tubulin acetylation with the K40R mutant inhibits migration

Reattachment is only one step in the metastatic cascade that could promote disseminated disease progression. Next, we investigated the role of acetylated α-tubulin in another metastatic process dependent upon cytoskeletal coordination, migration.

**Figure 4.**

α-Tubulin acetylation significantly affects reattachment rates of suspended breast tumor cells. A–C, real-time cell reattachment was analyzed using the xCELLigence system. Each cell line was plated in triplicate and error bars indicate ± standard deviation of those three wells. The graphs shown are representative of n = 3 independent runs/cell line. Cell Index represents the change in electrical impedance over time. A and B, representative graphs of suspended cell reattachment for stable pooled clones expressing the mutant K40R α-tubulin or the α-tubulin wild-type control. C, representative graph of MCF-7 cells transiently transfected with αTAT1-GFP or GFP control 24 hours before analysis.

MDA-MB-231 and BT-549 cell lines stably expressing the K40R α-tubulin mutant or the α-tubulin control were subjected to a real-time migration assay. This assay used plates similar to a Boyden chamber, where chemotaxis from the upper (serum-free) to the lower chamber (with serum) was monitored continuously. Over a period of 24 hours, both stable cell lines expressing the K40R α-tubulin mutant migrated significantly less, as compared with cells expressing the α-tubulin control (Fig. 5A and B). The underside of the upper chamber was stained at 24 hours to visualize cells that migrated toward the serum and representative images are shown.

MCF-7 cells transiently overexpressing αTAT1-GFP or the GFP control showed no significant difference in chemotaxis over 24 hours (Fig. 5C). These data support a model that transient increases in acetylated tubulin are not sufficient to promote migration in a nonmetastatic cell line, but acetylation of α-tubulin may be necessary for chemotaxis of more motile and metastatic breast tumor cells.

Acetylation of α-tubulin increases from primary tumor to metastasis in breast cancer patients

To extend the *in vitro* findings that tubulin acetylation promotes McTN generation, tumor cell reattachment, and affects migration, tubulin acetylation was examined in patient tumor samples. Tumor microarrays of primary lesions and matched lymphatic metastases of 144 patients with breast cancer were examined to determine whether changes in tubulin acetylation status from primary to metastasis could reflect a selective advantage for primary tumor cells during the metastatic process. Patient age and pathologic diagnosis can be viewed in Supplementary Fig. S4.

Immunohistochemistry was carried out for acetylated α-tubulin and each primary and metastatic tumor sample was blindly scored by a board-certified pathologist. The two-sided exact McNemar test for paired data revealed that based on the 144 samples, there is a statistically significant increase (P = 0.03) in acetylated α-tubulin score from the primary to matched metastasis in almost 30% of patients (42 total; Table 1). Seventeen patients also maintained high acetylation in their metastases, as compared with their primary tumor. Only 17% of patients (24 of 144) decreased in acetylation intensity, as compared with the 41% of patients (59 of 144) who increased or remained strongly positive from primary tumor to matched lymph node metastasis. Figure 6 shows representative images of matched tumor core samples probed for acetylated α-tubulin at ×8 and ×20 magnification. Each horizontal set of tumors represents matched primary and metastatic samples from a single patient.

**High α-tubulin acetylation is associated with the basal-like breast cancer subtype and patient survival**

The tissue microarray results demonstrate that acetylation of α-tubulin is a clinically relevant modification that increases in breast cancer metastases. Therefore, we investigated whether this modification could be linked to tumor characteristics in a larger patient cohort. Breast cancers can be molecularly classified by one of four subtypes: luminal A (LumA), luminal B (LumB), human epithelial growth factor receptor-2 positive (HER2), and basal-like (31, 32). These subtypes are associated with significantly different patient outcomes and can influence therapeutic strategies (33, 34). Basal-like breast cancers have poor patient prognosis...
with high metastasis rates, whereas the LumA subtype is associated with a better response to therapy and higher survival rates (35, 36). Given our previous findings that there is a strong correlation between high acetylation of α-tubulin and metastatic breast cancer cell lines as well as an increase in acetylation in patient metastases, we investigated the possibility that acetylation may be associated with more aggressive breast cancer subtypes. A primary tumor tissue sample set from the TCGA was probed for acetylated α-tubulin using the RPPA (37).

A large patient cohort (n = 392) representing all subtypes as well as a variety of ages and histologies was investigated (Supplementary Fig. S5A). When patients were separated by subtype, more than 72% (60 of 83) of patients diagnosed with basal-like breast cancer had high acetylated α-tubulin in their primary tumor (Fig. 7A). Only 25% of HER2 patients had high acetylation. The luminal subtypes showed a relatively even distribution between high and low acetylation intensity in the patients’ primary tumors (Fig. 7A). Basal-like tumors had an average acetylated α-tubulin intensity around 1.5-fold higher than the HER2 and luminal subtype tumors that was statistically significant (Fig. 7B).

Table 1. Acetylated α-tubulin is increased from patient primary to matched metastatic tumors

<table>
<thead>
<tr>
<th>Primary score</th>
<th>Low</th>
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<td>103</td>
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<tr>
<td>High</td>
<td>24</td>
<td>17</td>
<td>41</td>
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NOTE: Tumor scores for primary and matched lymph node metastases were compared for 144 patients. Tumor score of 0–1 is "Low"; 2–3 is "High" α-tubulin acetylation. The McNemar test was carried out and the two-sided exact test revealed that these rates were statistically different.

*P = 0.03.
It has been reported that 14 of the 392 patients in this sample set formed metastases after initial diagnosis, so we investigated these patients' primary tumor acetylation intensity. HER2 patients who formed metastases had low acetylation in the primary tumor and the luminal subtypes were split around the median. All patients with basal-like breast cancer who formed metastases had high α-tubulin acetylation intensity in their primary tumor (Supplementary Fig. S5B).

To investigate whether acetylated α-tubulin is associated with patient prognosis, the Kaplan–Meier analysis was used to estimate OS and PFS functions. Patients in this cohort (n = 277; Supplementary Table S1) were grouped using quartiles and separated into low, low-medium, medium-high, and high α-tubulin acetylation categories. The Cox regression model was used to assess the strength of association between OS (PFS) and acetylated tubulin intensity. The regression model indicated a trend toward increased risk of disease progression and death when compared with subjects with the lowest level of the marker. The estimated HR for PFS and OS are HR = 2.95 with the 95% CI, 1.07–8.12 (P = 0.036; Fig. 7C), and HR = 3.06 with the 95% CI, 0.98–9.52 (P = 0.04; Fig. 7D), respectively.

Discussion
This report investigates the specific role of α-tubulin acetylation at lysine 40 in the formation of suspended cell protrusions and tumor cell reattachment. Metastatic breast tumor cell lines have high acetylation of α-tubulin that is enriched in McTN protrusions upon suspension. Specifically reducing acetylation of α-tubulin with the K40R mutant significantly decreases McTN frequency in metastatic cell lines, while elevating acetylation via αTAT1 overexpression increases McTNs in a less aggressive cell line. Manipulation of acetylated α-tubulin through mutation and enzymatic regulation also demonstrates that suspended cell reattachment is dependent upon this modification. Stable McTNs have been associated with enhanced reattachment to endothelial monolayers (15) and CTC lung trapping in a murine metastasis model (7). Here, we find the specific PTM of α-tubulin acetylation enhances McTN

Figure 6. Acetylated α-tubulin is detected in patient primary and matched metastatic tumors. Representative images of matched patient primary invasive ductal carcinomas and lymph node metastases stained for acetylated α-tubulin. Horizontal pairings represent the primary tumor and the matched lymphatic metastasis from the same patient. Magnification image (×8) contains the tumor score (scale bar, 200 μm) and is compared with ×20 of the same sample (scale bar, 50 μm).
formation to promote suspended cell reattachment, one key step in the metastatic cascade.

Migration is another necessary step in cancer dissemination. We find that chemotaxis is significantly reduced with overexpression of the K40R α-tubulin mutant in metastatic breast tumor cell lines. This suggests acetylation may be necessary for proper chemotaxis in more invasive breast cancer cells. However, the role of stabilized microtubules in migration is greatly understudied, as compared with the more established role of actin. It has been shown that a reduction in acetylated α-tubulin impairs migration in neuronal cell lines (38, 39), but little is known about how it affects cancer cell motility. Conflicting reports have suggested the cytoskeletal alterations caused by HDAC inhibitors, specifically targeting HDAC6’s tubulin deacetylase activity, reduces cancer migration by increasing α-tubulin acetylation (22). However, effects of α-tubulin acetylation have been largely determined by nonspecific chemical inhibition or knockdown of deacetylases that also affect actin polymerization and severing (40, 41). Others have reported acetylated microtubules orient toward the leading edge of migrating MDA-MB-231 breast cancer cells to direct cell motility (42). Our results showing that α-tubulin acetylation enhances migration, McTN formation, and reattachment, indicate α-tubulin acetylation may actually promote a metastatic phenotype.

Our results indicate αTAT1 overexpression increases McTNs and reattachment in a nonmetastatic breast cancer cell line while others have shown αTAT1 enhances extracellular matrix invasion of the highly metastatic MDA-MB-231 cell line (42, 43). These studies suggest inhibition of αTAT1 could potentially impede reattachment and migration in tumors with high α-tubulin acetylation. However, there are no known inhibitors of αTAT1 for current investigative use. Until inhibitors are developed, insight can be gained from current tools that could affect α-tubulin acetylation. αTAT1 is thought to be the major TAT in vivo, but histone acetyltransferases (HAT) ELP3 (39) and Gcn5 (44) have also been shown to acetylate α-tubulin. Interestingly, overexpression of these enzymes are linked to progression of acute lymphoblastic leukemia (ALL; ref. 45), enhanced lung cancer growth (46), and increased motility in melanoma cells (47). HAT inhibitors...
have also been shown to have potent antitumor activity against triple-negative breast cancer xenografts in vivo (48). These targeted therapies are mainly aimed at inhibiting HAT activity but should be further investigated to determine whether HAT inhibition could also reduce tubulin acetylation at clinically tolerable levels. This dual mechanism of action may prove to be more efficacious in certain cancers, specifically those with high acetylation of α-tubulin.

Our current results indicate α-tubulin acetylation is increased from the primary tumor to the matched lymph node metastasis in 30% of patients (42 of 144) while high acetylation was detected in 41% of screened breast cancer metastases (59 of 144). The dependence upon endogenous acetylation for reattachment and chemotaxis of metastatic breast tumor cell lines and the significant increase of α-tubulin acetylation in metastatic tumors support a model in which tubulin acetylation confers a selective advantage for metastatic potential. This model is further supported by very recent evidence that high α-tubulin acetylation is associated with a more invasive phenotype in human pancreatic cancer cell lines (49) as well as lymph node metastasis and poor prognosis in patients with head and neck squamous cell carcinoma (50).

We report for the first time that α-tubulin acetylation is also associated with the basal-like breast cancer subtype in patients. Basal-like breast cancers are negative for estrogen receptor (ER)- or progesterone receptor (PR)-responsive genes and genes associated with HER2 amplification (31). This aggressive subtype is correlated with increased risk of metastatic spread and poor patient prognosis (33, 36). Although there is consensus for what characteristics basal-like tumors lack (ER/PR/HER2 amplification), there are very few positive markers that can define the basal-like subtype (36). We found that more than 72% of basal-like tumors had high α-tubulin acetylation. The average acetylated tubulin intensity for basal-like tumors was approximately 1.5 times higher than the HER2 and luminal subtype tumors, and all basal-like primary tumors that metastasized had high α-tubulin acetylation. Our results also indicate high α-tubulin acetylation in the primary tumor is significantly associated with an increased risk of disease progression and death in non-HER2 patients. We believe this could be due to an inverse relationship between α-tubulin acetylation and HER2, because previous studies have shown that HER2 overexpression reduces α-tubulin acetylation (51, 52). Additional studies using a larger patient cohort and further investigation into the inverse relationship within the HER2 subtype are necessary to determine the clinical significance of α-tubulin acetylation as a patient prognostic factor.

Despite the need for treatments to prevent metastatic dissemination, there are a number of challenges in developing such therapies. One major challenge in targeting metastasis is that tumor cells can leave the primary site and begin metastatic progression before cancer is clinically detected (53). Although CTCs can enter the bloodstream before diagnosis, early steps of the metastatic cascade are still reasonable targets for therapeutic intervention. Tumor cells have been shown in animal models to reenter the circulation and seed other metastatic sites as well as self-seed the primary tumor throughout cancer progression (54, 55). Surgery to remove a primary tumor seeds millions of cells into the patient’s bloodstream (56) and CTCs can be detected in a patient’s blood for years after primary tumor resection (57). Antimetastatic therapies may be most effective before surgery on the primary tumor or in patients at high risk of disseminated disease (55). A key advantage of targeting disseminated and circulating cells is that they are more accessible for chemotherapeutics in the bloodstream (55). Ideally, targeted therapies against dissemination, reattachment, or invasion could be successfully combined with existing cancer treatments to control or prevent metastatic disease (10, 55).

The current results define a novel mechanism in which acetylation of lysine 40 of α-tubulin promotes MetTN generation, tumor cell reattachment, and chemotaxis that are selective advantages for metastatic potential and particularly enriched in basal-like breast cancers. The resulting opportunity for α-tubulin acetylation to serve as both a diagnostic and therapeutic target for metastatic breast cancer will be an important avenue of ongoing investigation.

Disclosure of Potential Conflicts of Interest

R.A. Whipple has ownership interest (including patents) in a patent. G.B. Mills reports receiving a commercial research grant from AstraZeneca, HanAl Bio, and ClasnoSmithKline; has ownership interest (including patents) in Catena Pharmaceuticals, PIV Ventures, Spindle Top Ventures, and is a consultant/advisory board member for AstraZeneca, Bind, Critical Outcome Technologies, HanAl Bio Korea, Naevolution, and Symphogen. S.S. Martin has ownership interest (including patents) in a University of Maryland patent. No potential conflicts of interest were disclosed by the other authors.

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α-Tubulin Acetylation Elevated in Metastatic and Basal-like Breast Cancer Cells Promotes Microtentacle Formation, Adhesion, and Invasive Migration

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