Cortactin Overexpression Regulates Actin-Related Protein 2/3 Complex Activity, Motility, and Invasion in Carcinomas with Chromosome 11q13 Amplification

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Abstract
Carcinoma cell motility and invasion are prerequisites for tumor cell metastasis, which requires regulation of the actin cytoskeleton. Cortactin is an actin-related protein 2/3 (Arp2/3) complex–activating and filamentous (F)-actin–binding protein that is implicated in tumor cell motility and metastasis, partially by its ability to become tyrosine phosphorylated. Cortactin is encoded by the CTTN gene and maps to chromosome 11q13, a region amplified in many carcinomas, including head and neck squamous cell carcinoma (HNSCC). CTTN gene amplification is associated with lymph node metastasis and poor patient outcome, and cortactin overexpression enhances motility in tumor cells lacking 11q13 amplification. However, a direct link between increased motility and invasion has not been reported in tumor cells with chromosome 11q13 amplification and cortactin overexpression. In this study, we have examined the relationship between CTTN amplification and tumor cell motility in HNSCC. In 11 of 39 (28%) HNSCC cases, cortactin overexpression determined by immunohistochemistry correlates with lymph node metastasis and CTTN gene amplification. HNSCC cells containing cortactin gene amplification and protein overexpression display increased binding and activation of Arp2/3 complex, and were more motile and invasive than HNSCC cells lacking CTTN amplification. Down-regulation of cortactin expression in CTTN-amplified HNSCC cells by small interfering RNA impairs HNSCC motility and invasion. Treatment of HNSCC cells with the epidermal growth factor receptor inhibitor gefitinib inhibits HNSCC motility and invasion.

Introduction
Tumor cell motility is required for local-regional invasion and distant metastasis. Motility is initiated by protrusion of the leading edge of the cell, resulting in the production of polarized lamellipodia that are oriented toward the direction of movement. Production and extension of lamellipodia occurs in response to a wide variety of extracellular ligands that signal through transmembrane receptors (1). Lamellipodia serve to orient tumor cells toward the vasculature in response to chemotactic agents and are required for tumor cell invasion (2).

Chemotactic receptor activation results in increased Rho GTPase and Src family kinase activity, which in turn leads to the activation of Wiskott-Aldrich syndrome proteins (WASp) that drive lamellipodia protrusion (3). Activation of WASps by GTPase activation or tyrosine phosphorylation exposes a cryptic carboxyl-terminal domain containing an acidic region that binds and activates the actin-related protein 2/3 (Arp2/3) complex. Arp2/3 complex activity results in nascent polymerization of actin filaments at the cell periphery, providing the protrusive force responsible for lamellipodia extension at the leading edge (4). Regulation of the actin cytoskeleton by Arp2/3 complex activity has been proposed as a mechanism for controlling tumor cell migration, invasion, and metastasis (5).

In addition to WASps, another protein that binds and activates Arp2/3 complex is cortactin, a Src kinase substrate and filamentous (F)-actin–binding protein that is enriched in lamellipodia (6, 7). Cortactin consists of an amino-terminal acidic domain followed by a repeat region, a proline-rich region containing sites of tyrosine phosphorylation, and a distal Src homology 3 (SH3) domain that interacts with a variety of proline-rich proteins (6, 7). Cortactin directly activates Arp2/3 complex actin nucleation activity through the binding of the amino-terminal acidic domain region to Arp2/3 complex and the repeat region to F-actin (7). Nucleation activity and lamellipodia protrusion is further enhanced by the binding of the SH3 domain to dynamin 2 or WASp-interacting protein (8, 9). The cortactin SH3 domain also binds and activates neuronal (N)-WASp, which in turn promotes Arp2/3 actin nucleation and cell motility (10, 11). Additionally, cortactin modulates cell movement by the phosphorylation of three specific tyrosine residues (421, 470, and 486 in humans) by Src and other oncogenic tyrosine kinases (12). Tyrosine-phosphorylated cortactin is found in lamellipodia (13) and correlates with enhanced cell motility and tumor cell metastasis in nude mice (14). Other functions of cortactin include stabilization of Arp2/3–F-actin networks and participation in receptor-mediated endocytosis (15, 16).

Genomic instability is a hallmark of cancer progression and results in the accumulation of specific genetic events. Amplification of CTTN is found in lamellipodia (13) and correlates with enhanced cell motility and tumor cell metastasis in nude mice (14). Other functions of cortactin include stabilization of Arp2/3–F-actin networks and participation in receptor-mediated endocytosis (15, 16).
of chromosome 11q13 is found in several tumors and is one of the most prevalent genetic alterations in head and neck squamous cell carcinoma (HNSCC), occurring in approximately one third of all cases (17). Amplification of 11q13 correlates with several important clinical variables, including increased invasive disease, regional metastasis, more frequent recurrence, and increased disease-specific mortality (18). The human cortactin locus CTTN (formerly designated EMS1) is located within the 11q13 region and cortactin overexpression resultant from gene amplification is found in HNSCC and other carcinomas (19, 20). CTTN amplification and overexpression directly correlates with poor outcome in HNSCC patients (21). Based on these data, it has been proposed that cortactin is the critical gene in the amplified chromosome 11q13 region responsible for enhancing carcinoma motility and invasion (22).

Indirectly supporting this, several recombinant overexpression studies in cells lacking 11q13 amplification have established cortactin as a key regulator in migration, invasion, and metastasis (12, 23, 24). To determine the role cortactin plays in motility and invasion in the context of tumors with 11q13 amplification, we have investigated the effect of cortactin overexpression in HNSCC cells with and without 11q13 amplification and have determined that cortactin overexpression derived from 11q13 amplification results in increased Arp2/3 complex activity, tumor cell motility, and invasion. We also show that cortactin tyrosine phosphorylation is required for efficient HNSCC migration, and that inhibition of cortactin tyrosine phosphorylation with the targeted therapeutic drug gefitinib reduces HNSCC motility and corresponds with drug gefitinib reduces HNSCC motility and corresponds with decreased cortactin tyrosine phosphorylation. These results indicate that cortactin directly contributes to elevated motility and invasion in HNSCC tumors with 11q13 amplification by increasing Arp2/3 actin nucleation activity, and that cortactin tyrosine phosphorylation may serve as a potential diagnostic marker for validation of targeted therapeutic agents against tyrosine kinase pathways involved in tumor cell migration and invasion.

Materials and Methods

Tumor samples. Formalin-fixed, paraffin-embedded tissue blocks from 10 cases of HNSCC were retrieved from the University of Colorado Health Sciences Center Department of Pathology archives. All tissue and case report information was used under approval from the Colorado Multiple Institutional Review Board. An additional 29 HNSCC cases were analyzed from a HNSCC tissue microarray (YTM4) constructed at Yale University Department of Pathology.

Immunohistochemistry. Five-micrometer sections cut from tissue blocks and the HNSCC microarray were processed for immunohistochemical labeling as described (25), except that sections were incubated with 0.4% pepsin for 10 minutes at 37°C for antigen retrieval. The pan-specific cortactin monoclonal antibody 4F11 and avian-specific control cortactin antibody 1H3 (26) were used at 1 µg/mL. Samples were visualized with an Olympus AX70 microscope and images were acquired using the Micro-BrightField system (Williston, VT).

Fluorescence in situ hybridization. Dual-color fluorescence in situ hybridization (FISH) analysis was conducted to determine CTTN copy numbers in tumor specimens and in HNSCC cell lines. Two P1 genomic cortactin clones (20) were used in combination with a control probe for the centromeric region of chromosome 11 (CEP 11 SpectrumGreen, Vysis/Abbott Molecular, Des Plaines, IL). The CTTN P1 clones were labeled by nick translation with dUTP-conjugated Spectrumred, combined with the SpectrumGreen CEP 11, and hybridized as described (27).

Tumor specimens were quantitated for CTTN gene amplification by epifluorescence, with cases containing a ratio of >2 CTTN/CEP 11 copies per nucleus considered amplified. For determining cortactin gene amplification in HNSCC cell lines, mitotic arrest was achieved by treating the cells with colcemid (0.05 µg/mL) for 2 hours and chromosome elongation by treatment with ethidium bromide (5 µg/mL) for 1 hour. Cells were trypsinized, lysed with 75 mmol/L potassium chloride, and fixed with 3:1 (v/v) mixture of methanol and acetic acid. Cells were dropped on glass slides and slides were hybridized and processed as described (28). The number of CTTN and CEP 11 probe signals were determined for a minimum of 100 nuclei in each cell line by epifluorescence microscopy.

Cell lines, transfection, and Western blotting. HNSCC lines UMSCC2, UMSCC8, UMSCC10A, UMSCC19, and HN4 were maintained in DMEM (Mediatech, Herndon, VA) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT), 50 µg/mL penicillin-streptomycin, and nonessential amino acids. The MSK921 and 584 lines were cultured in l-glutamine–free RPMI (Mediatech) supplemented with 10% FBS and 50 µg/mL penicillin-streptomycin. For transfection, 106 cells were incubated with 2 µg FITC-labeled control small interfering RNA (siRNA); siRNA targeted against human cortactin (Qiagen, Valencia, CA); pEGFPN1 green fluorescent protein (GFP) vector (BD Clontech, Palo Alto, CA); GFP-cortactin (9); FLAG-wild type or FLAG-Y421F, FLAG-Y466F, FLAG-Y482F (triple point mutant (TPM)) cortactin (13) in solution V (Amaya Biosystems, Berlin, Germany); and transfected with the Nucleofector I device (Amaya Biosystems) according to the instructions of the manufacturer. Cells were plated and allowed to recover for 24 to 48 hours before use in motility, invasion, or Western blot experiments. Cell lysis and Western blot analysis was done as previously described (13). The following antibodies and final concentrations were used: 4F11 (0.5 µg/mL), anti-ARPC2 (p34 subunit of Arp2/3 complex, 1:1,000, Upstate, Waltham, MA), anti-α-actinin (1:5,000, Calbiochem, La Jolla, CA), Living Colors anti-GFP clone J-8 (1:1,000; BD Clontech), and antiphospho-tyrosine 4G10 (1:1,000; Upstate). Scanned blots were quantified using ImageQuant TL software (Amersham Biosciences, Piscataway, NJ). Blots were done in triplicate.

Arp2/3 pulldown and actin incorporation assays. The recombinant glutathione-S-transferase (GST)-verprolin cofillin homology acidic (VCA) domain of N-WASP was expressed and purified using standard techniques. Cell lysates (1 mg) were incubated with 50 µg GST-VCA prebound to 40 µL glutathione-agarose (50% slurry; Pierce, Rockford, IL) for 3 hours at 4°C, washed, and processed for Western blotting with 4F11 and anti-ARPC2 antibodies. Assays were done in triplicate. Globular (G)-actin incorporation (barbed end) assays on serum-starved cells were done essentially as described (29), except Alexa–888–G–actin (Invitrogen, Carlsbad, CA) was used and cells were stimulated for 10 minutes with 100 ng/mL epidermal growth factor (EGF; Upstate). Fixed cells were imaged with a Nikon TE2000 epifluorescence microscope equipped with a CoolSnap i/x charge-coupled device camera (Roper Scientific, Tucson, AZ). Quantification of actin incorporation was done using a script written in the Metamorph v6.0 software suite (Molecular Devices, Downingtown, PA). The script identifies peripheral areas around the cell with pixel intensities above background and creates a 3-pixel-thick ring over the regions of circumferential fluorescent actin incorporation. Border widths were adjusted manually as required to compensate for varying degrees of cortical actin thickness. Quantification of G-actin incorporation was taken as the total fluorescence intensity divided by the surface area of the measured region. Fifty EGF-stimulated and nonstimulated cells for each line were analyzed in three independent experiments.

Cell motility and invasion assays. Transwell cell migration and Matrigel-coated invasion assays were conducted using 8 µm pore size chambers (Fisher Scientific, Pittsburgh, PA and BD Transduction, San Jose, CA) essentially as described (30). Cells (105) were added to each upper chamber and cells were allowed to migrate or invade for 24 hours. In some experiments, 1 µmol/L gefitinib (Iressa, AstraZeneca, Macclesfield, Chesh-ire, United Kingdom) was added to both chambers at the beginning of the experiment. Fixed cells were stained with 3 µg/mL 4,6-diamidino-2-phenylindole (DAPI) and motility was assessed by counting nuclei in four random microscopic fields through the use of an automated Metamorph 2.0 script that accurately determines cell count by delineating cell shapes and dividing by the average nuclear surface area. For siRNA rescue experiments, cell motility was measured using electric substrate impedance sensing...
(Applied BioPhysics, Troy, NY), as described (31), using 40 kHz voltage pulse for initial cell ablation and 10 ng/mL EGF as the motile stimulus. Impedance was measured for 24 hours. A minimum of three independent experiments were done in all assays.

Statistical analysis. P values were determined by the unpaired, two-tailed, Student’s t test.

Results

Cortactin overexpression in invasive HNSCC. Although cortactin gene amplification and transcription has been documented with PCR-based methods in HNSCC and correlates with poor prognosis (21), cortactin localization and CTTN amplification status in HNSCC have not been visualized and correlated to invasive capacity. To address this, we analyzed cortactin expression in 39 cases of primary HNSCC by immunohistochemistry and CTTN amplification by FISH analysis from archived tissue sections and a HNSCC tissue microarray (Fig. 1). In 11 cases (28%), cortactin overexpression was detected in the tumor cell cytoplasm, with marked enrichment of the protein at the cell periphery and at the leading edge of cells in tumors that displayed local-regional invasion and cervical lymph node metastasis (Fig. 1A). Serial sections treated with a monoclonal antibody specific to avian cortactin as a control were not significantly stained (Fig. 1A). All tumors that overexpressed cortactin also contained CTTN amplification as determined by FISH (Fig. 1B). Noninvasive, node-negative tumors had little cortactin staining and did not contain CTTN amplification (Fig. 1). These data indicate that cortactin overexpression and gene amplification in HNSCC correlates with an invasive phenotype.

Cortactin overexpression and gene amplification correlate in HNSCC cells. To determine the relationship between cortactin overexpression and gene amplification in HNSCC cells, we quantified cortactin protein and gene levels in several HNSCC cell lines where cortactin expression and/or chromosome 11q13 amplification status had been partially defined (Fig. 2; refs. 32–34). Cortactin protein and gene levels were standardized against UMSSC10A, a line normal for cortactin expression compared with nontransformed epithelial cells (not shown) and disomic for CTTN. Western blot analysis indicated a 6- to 12-fold increase in cortactin expression for lines UMSSC19, UMSSC2, and MSK921 (Fig. 2A and B). The HN4 line had a 2-fold increase in cortactin expression, whereas UMSSC8 and UMSSC84 contained normal cortactin levels (Fig. 2A and B). The amount of Arp2/3 complex for each line was also determined with an antibody against the ARP C2 complex subunit. In all lines, no significant differences in Arp2/3 expression were detected (Fig. 2A). Cytogenetic analysis in metaphase spreads indicated that these lines were hypo-diploid to near-triploid for CTTN. Quantification of the CTTN locus by FISH indicated that UMSSC19, UMSSC2, and MSK921 contained clustered CTTN amplification, with mean copy numbers ranging from 8 to over 20 per cell (Fig. 2C). All other lines had on average two to four CTTN copies per cell. With the exception of the HN4 line, the level of cortactin protein expression directly correlates with CTTN copy number in each line (Fig. 2B and C). These data indicate that cortactin expression and gene levels are tightly coupled, and that amplification of CTTN correlates with cortactin overexpression in HNSCC.

HNSCC cells containing cortactin overexpression have increased Arp2/3 binding and activation. Our findings that cortactin is enriched at the cell periphery when overexpressed in invasive tumors and that Arp2/3 levels are unchanged suggests that cortactin overexpression resulting from chromosome 11q13 amplification may increase the amount of cortactin associated with Arp2/3 complex, resulting in enhanced Arp2/3 actin nucleation activity. To test this possibility, we conducted Arp2/3 pulldown experiments from HNSCC cells with and without cortactin overexpression using a N-WASP recombinant GST-VCA domain, a region of N-WASP that simultaneously binds with cortactin to different subunits of Arp2/3 (35). A 1.5- to 2-fold increase in cortactin binding to Arp2/3 complex was observed in cell lines UMSSC2 and UMSSC19 that overexpress cortactin compared with UMSSC10A and UMSSC8 (Fig. 3A). A similar increase in binding was observed in the overexpressing line MSK921 (data not shown). These results suggested that the increased association between Arp2/3 and cortactin may result in increased cortactin-mediated Arp2/3 actin nucleation activity. To test this, serum-starved HNSCC cells were permeabilized with saponin in the presence of Alexa-488–G-actin and then stimulated with EGF to allow the incorporation of labeled actin into nascent filament ends (Fig. 3B and C). This method allows for determining the degree of Arp2/3 actin-nucleation activity within newly forming lamellipodia downstream of EGF receptor (EGFR) activation (36). In all HNSCC lines examined, maximal incorporation of Alexa-488–G-actin into the
peripheral cell edge occurred 10 minutes after EGF stimulation, resulting in circumferential rings of varying fluorescence intensities (Fig. 3B). Levels of EGFR were similar in all lines examined (data not shown). Quantitative microscopic analysis of this region for the 

**Figure 2.** Determination of cortactin protein and CTTN levels in HNSCC cell lines. A, Western blot analysis of cortactin and Arp2/3 complex expression. Equal amounts of cell extracts from HNSCC cell lines (top) were subjected to SDS-PAGE and Western blotted with antibodies against cortactin, against the ARPC2 subunit of Arp2/3 complex, and against β-actin as a loading control. B, quantification of protein levels in HNSCC cell lines. Western blots described in (A) were scanned and quantified with the cortactin/β-actin values (designated as 1.0) for control purposes; bars, SE. *, P < 0.05. C, CTTN levels in HNSCC cell lines. Fixed cells were analyzed by FISH using a combination of two independent probes containing genomic CTTN sequences conjugated to SpectrumGreen. Columns, averaged values normalized to the UMSCC10A cortactin/β-actin values (designated as 1.0) for control purposes; bars, SE. *, P < 0.001.

MSK921 and UMSCC19 lines showed a nearly 2-fold increase in cortical actin polymerization compared with the UMSCC8 and 584 lines (Fig. 3C). Similar results were observed in UMSCC2 cells, although precise localization of labeled actin within lamellipodia was difficult to discern in this cell line (data not shown). These data indicate that HNSCC cells with cortactin overexpression are enhanced in their ability to bind and activate Arp2/3 complex at the cell periphery.

**HNSCC cells with 11q13 amplification display increased cell motility and invasion.** To determine if HNSCC cells with 11q13 amplification and cortactin overexpression display increased cell motility and invasion, transwell migration and Matrigel invasion assays were conducted (Fig. 4). No significant changes in cell division rates were observed between all lines during the assay, allowing for direct comparisons to be made. Two different chemotax attractants, EGF and FBS, were evaluated because all HNSCC lines used overexpress EGFR (except UMSCC10A) and EGFR overexpression is clinically known to correlate with increased HNSCC motility and invasion (37). Increased migration and invasion was observed with UMSCC19, UMSCC2, and MSK921 cells toward 10 ng/mL EGF compared with all other lines tested (Fig. 4). Similar results were obtained when 10% FBS was used as the chemotax attractant, with some variability in the motility and/or invasive response observed in the UMSCC8 and MSK921 lines when compared with EGF (Fig. 4). Collectively, these data suggest that 11q13 amplification and cortactin overexpression correlates with increased HNSCC motility and invasion.

**Cortactin directly modulates HNSCC motility and invasion.** The 11q13 locus is a gene-rich region that upon amplification may express proteins besides cortactin that can contribute to cell motility and invasion in HNSCC. To determine the contribution of cortactin in HNSCC motility and invasion, cortactin expression was down-regulated in HNSCC cells with 11q13 amplification by transfection with a cortactin-specific siRNA. The degree of cortactin knockdown varied between the different cell lines tested, ranging to near-complete knockdown for UMSCC2 (97%), 83% for UMSCC19, and 40% for MSK921 (Fig. 5A). This variation is reflective of differing transfection efficiencies among the tested lines as determined by control transfections with a plasmid encoding GFP (data not shown). UMSCC2 cells were inhibited in motility 47% toward EGF and 43% serum when compared with internal, cell line–specific controls (Fig. 5B). The corresponding values for UMSCC19 were 51% and 52% for MSK921, the values were 34% and 9%, respectively. Invasion was also inhibited in cells with cortactin knockdown, with a 29% reduction for UMSCC2, 21% for UMSCC19, and 10% for MSK921 with EGF and the motile stimulus. These values were 43%, 65%, and 14%, respectively, when FBS was used (Fig. 5B). To further confirm that cortactin expression levels influence HNSCC motility and invasion, UMSCC8 and HN4 cells (lines without 11q13 amplification) were transfected with a construct expressing either GFP or GFP-cortactin. Expression of GFP-cortactin in these cells raised the total cortactin levels to 125% in UMSCC8 and 140% in MSK921 with EGF and the motile stimulus. These values were 43%, 65%, and 14%, respectively, when FBS was used (Fig. 5B).

***Gefitinib inhibits HNSCC motility and down-regulates cortac-**

...tactin tyrosine phosphorylation. Cortactin tyrosine phosphorylation...
The ability of gefitinib to suppress HNSCC motility and cortactin tyrosine phosphorylation suggested that cortactin tyrosine phosphorylation contributes to HNSCC motility. To directly assess this, cortactin expression in UMSCC2 cells was reduced by siRNA and expression was restored by transfection of either wild-type FLAG-tagged murine cortactin or with a FLAG-cortactin construct containing phenylalanine to tyrosine point mutations at the Src-targeted codons 421, 466, and 482 (FLAG-TPM cortactin), previously shown to suppress cell motility (12). Overexpression of FLAG-wild type cortactin in UMSCC2 cells without cortactin knockdown led to a 260% increase in cell migration compared with control-transfected cells (Fig. 6D). This increase in motility is likely due to the additional high amount of recombinant murine cortactin expression driven by the cytomegalovirus promoter present in the recombinant cortactin constructs (data not shown). Using these cells as the control for recombinant murine cortactin expression, expression of FLAG-wild type murine cortactin following siRNA-mediated knockdown of endogenous cortactin expression resulted in a 140% reduction in cell migration compared with FLAG-wild type cortactin–overexpressing cells, reflecting the contribution of endogenous cortactin expression on UMSCC2 migration. Expression of Flag-TPM cortactin in UMSCC2 cells following cortactin knockdown reduced migration 261% compared with the overexpression control, 121% compared with cells with FLAG-wild-type cortactin overexpression in the presence of endogenous cortactin knockdown, and 99% of the transfection
control (Fig. 6D). Direct comparison between FLAG-wild type and FLAG-TPM cortactin motility in cells with endogenous cortactin knockdown indicates that FLAG-TPM cells migrate at 45% of the level of FLAG-wild type cortactin–expressing cells. These data indicate that cortactin phosphorylation at tyrosines 421, 466, and 482 is responsible for 55% of the motility ascribed to cortactin in the cell line used. These data are consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation in the lamellipodia of cells with suppressed cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with the level of EGFR gene expression.

Discussion

Studies to date have shown that cortactin overexpression resulting from 11q13 amplification in HNSCC and other carcinomas corresponds with poor patient outcome, and that ectopic overexpression of cortactin in cancer cells lacking 11q13 amplification increases tumor cell motility, invasion, and metastasis. Our results directly show that cortactin overexpression resulting from 11q13 and corresponding CTTN amplification in carcinomas leads to increased tumor cell motility and invasion. In addition, our data indicate that these effects are likely due to increased binding between cortactin and Arp2/3 complex, potentially serving to enhance Arp2/3 actin nucleation activity within tumor cell lamellipodia. The resulting enhancement of lamellipodia protrusion would allow tumor cells to become more motile, increasing their ability to undergo local-regional invasion, invasation and extravasation, and distant metastasis. The corresponding ability of the EGFR inhibitor gefitinib to suppress HNSCC motility and cortactin tyrosine phosphorylation indicates that tyrosine phosphorylation of cortactin may also serve as a valid therapeutic end point marker for metastatic efficacy in the treatment of HNSCC patients with inhibitory compounds targeting the EGFR pathway.

In agreement with previous work (19, 21), our results show that chromosome 11q13 amplification corresponds with cortactin overexpression in all examined HNSCC cases, correlating with tumor invasion and lymph node metastasis. The enrichment of cortactin within lamellipodia in invasive HNSCC cases is likely reflective of a role in enhancing tumor cell motility, as evidenced by the increased motility and invasion of HNSCC lines with cortactin overexpression. Moreover, in HNSCC cells, the degree of cortactin overexpression corresponds with the level of CTTN amplification, with the greater degree in HNSCC cell lines with the highest CTTN amplification indicating that cortactin phosphorylation at tyrosines 421, 466, and 482 is responsible for 55% of the motility ascribed to cortactin in the cell line used. These data are consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with the level of EGFR gene expression.


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In agreement with previous work (19, 21), our results show that chromosome 11q13 amplification corresponds with cortactin overexpression in all examined HNSCC cases, correlating with tumor invasion and lymph node metastasis. The enrichment of cortactin within lamellipodia in invasive HNSCC cases is likely reflective of a role in enhancing tumor cell motility, as evidenced by the increased motility and invasion of HNSCC lines with cortactin overexpression. Moreover, in HNSCC cells, the degree of cortactin overexpression corresponds with the level of CTTN amplification, with the greater degree in HNSCC cell lines with the highest CTTN amplification indicating that cortactin phosphorylation at tyrosines 421, 466, and 482 is responsible for 55% of the motility ascribed to cortactin in the cell line used. These data are consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with our finding of elevated actin incorporation (via the production of free barbed ends) in HNSCC patient lines with elevated cortactin expression, consistent with the level of EGFR gene expression.

Concurrent with its role in cell migration, evidence to date indicates that cortactin also regulates tumor cell invasion. In many carcinomas, cortactin is enriched within invadopodia, protrusive membranous structures in tumor cells that emanate from the ventral cell surface that regulate extracellular matrix degradation and protrusion (40). Recent studies have shown that cortactin is required for invadopodia formation and is essential for the recruitment of membrane type 1 matrix metalloproteinase, the main enzyme required for invadopodia gelatinase activity, to invadopodia (41). The ability of cortactin to regulate N-WASP and Arp2/3 activity is likely responsible in part for invadopodia formation because invadopodia require the activity of both of these molecules to form and extend (42). In addition to regulating invadopodia protrusion, cortactin may also play an important role in the endocytosis of proteolyzed matrix components as evidenced by the requirement of cortactin SH3 domain ligand dynamin 2 for invadopodia-induced matrix degradation (43).

Although our data indicates that down-regulation of cortactin expression with siRNA in tumor cells with CTTN amplification significantly decreases carcinoma motility and invasion, it is
possible that other genes within the 11q13 amplicon are overexpressed that may also play a role in regulating cell migration and invasion. For example, the locus for the serine/threonine kinase Pak1 maps to the 11q13 region, and Pak1 overexpression has been documented in invasive HNSCC and breast carcinoma (44). Pak1 phosphorylates cortactin and is associated with lamellipodia formation in platelets (45). Pak1 also phosphorylates and activates Arp2/3 complex, inducing lamellipodia extension and cell motility (46). Pak1 may therefore serve to regulate carcinoma motility and invasion directly through Arp2/3 complex and indirectly through cortactin. Additionally, other genes yet to be characterized within the 11q13 amplicon may also contribute to cell motility.

Figure 5. Modulation of cortactin expression in HNSCC cells directly affects motility and invasion. A, down-regulation of cortactin in overexpressing HNSCC cells. Top, cells were transfected without RNA (mock), with cortactin siRNA (siRNA), or with a control siRNA conjugated to FITC (FITC-ctl). After 48 hours, cells were lysed and 50 mg total cell protein were assayed by Western blot analysis for knockdown of cortactin expression with anticoartactin antibodies. Blots were stripped and reprobed with antibodies against β-actin as a loading control. Bottom, degree of cortactin knockdown in siRNA-treated cells was quantified by densitometry and expressed relative to cortactin levels in cells transfected with FITC-conjugated control siRNA. B, cortactin knockdown in overexpressing HNSCC cells impairs cell motility and invasion. HNSCC cells 48 hours after transfection with cortactin siRNA were assayed for transwell motility (top) and invasion with 10 ng/mL EGF or 10% FBS as mitogenic stimuli (bottom). Columns, percentage motility or invasion compared with each corresponding line transfected with FITC-conjugated control siRNA (control, 100%); bars, SE for a minimum of three independent experiments. *, P < 0.05. C, ectopic cortactin overexpression in HNSCC cells. HNSCC cells with normal cortactin expression (UMSCC8 and HN4) were transfected with GFP-cortactin or GFP control constructs for 24 hours and cell lysates were analyzed by Western blot analysis with an anti-GFP monoclonal antibody (top). Blots were stripped and subsequently probed with cortactin and β-actin antibodies. The positions of GFP-cortactin, GFP, and endogenous cortactin are indicated (right). Bottom, scanned blots were quantified for GFP-cortactin and endogenous cortactin expression, and the degree of total cortactin overexpression is shown relative to GFP transfected controls. D, ectopic cortactin overexpression increases HNSCC motility and invasion. UMSCC8 and HN4 cells expressing GFP or GFP-cortactin were analyzed for motility and invasion as in (B). Columns, percentage motility or invasion compared with each corresponding line transfected with GFP (control, 100%); bars, SE for a minimum of three independent experiments.
CTTN may be the predominant 11q13 gene involved in regulating carcinoma invasion, it is likely that 11q13 amplification exerts a pleiotropic effect on tumor cell motility and invasion through the overexpression of several gene products.

Consistent with previous work (44), gefitinib inhibits motility in all HNSCC lines analyzed in this study that overexpress EGFR. Moreover, our data indicate that cortactin tyrosine phosphorylation is down-regulated in HNSCC cells treated with gefitinib, indicating that the EGFR-Src signaling pathway is used for Src activation and subsequent cortactin tyrosine phosphorylation in HNSCC. Direct evaluation of cortactin tyrosine phosphorylation on HNSCC motility by expression of recombinant phosphorylation-deficient cortactin in cells lacking endogenous cortactin reduced HNSCC migration levels by 55%, clearly indicating that tyrosine phosphorylation of cortactin provides a substantial contribution to HNSCC migration. Whereafter gefitinib largely inhibited motility (>90%) in response to EGFR regardless of 11q13 amplification and cortactin expression status, the ability of gefitinib to inhibit cell migration in response to serum, a more physiologically relevant stimulus, was reduced in cells with 11q13 amplification and cortactin overexpression. The HNSCC cell lines used in this study show an inverse dose-response relationship between cortactin expression levels and cell motility in the presence of gefitinib. One explanation for this can be derived from a recent study that showed reduced ligand-induced EGFR internalization, ubiquitylation, and receptor degradation in HNSCC cells containing cortactin overexpression (47). The subsequent persistence of EGFR in internalized endosomes in HNSCC cells with cortactin overexpression may provide a sustained counteractive effect to gefitinib, especially in HNSCC cases with EGFR overexpression, because endosomal EGFR is still capable of activating Src (48) and therefore would promote cortactin phosphorylation. Alternatively, multiple pathways besides EGFR induce cortactin tyrosine phosphorylation (6), potentially allowing HNSCC cells to circumvent gefitinib-blocked EGFR and lead to cortactin tyrosine phosphorylation. The fact that gefitinib does not completely abolish cortactin tyrosine phosphorylation in HNSCC (Fig. 6C) supports these hypotheses.

The resistance of serum-stimulated HNSCC cells with cortactin overexpression to the antimitotic effects of gefitinib is consistent with similar findings in HNSCC cells where cyclin D1–overexpressing lines were resistant to the antiproliferative effects of gefitinib (49). Because cyclin D1 and cortactin are often coamplified and overexpressed in HNSCC (18), it is possible that the two effects are linked, and that the overexpression of genes resulting from chromosome 11q13 amplification in HNSCC and other carcinomas may have additive effects that lead to gefitinib resistance. This might partially explain the modest success observed in phase II clinical trials with gefitinib in HNSCC (50). Such results may support validation of cortactin or other genes overexpressed from the 11q13 amplicon as potential prognostic or therapeutic biomarkers for HNSCC and other carcinomas as predictors of tumor cell growth, motility, and invasion.

**Figure 6.** Gefitinib inhibits HNSCC motility and cortactin tyrosine phosphorylation. HNSCC cells were assayed for transwell motility toward 10 ng/mL EGF (A) or 10% FBS (B) in the absence or presence of 1 mmol/L gefitinib for 24 hours (top). Bars, SE for a minimum of three independent experiments. *P < 0.05. The percentage motility for gefitinib-treated cells is shown relative to untreated control cells (bottom). C, gefitinib impairs cortactin tyrosine phosphorylation in HNSCC cells. Cortactin immunoprecipitated from HNSCC cells treated without or with 1 mmol/L gefitinib for 4 hours was assayed for tyrosine phosphorylation (pTyr, top) and cortactin expression (bottom) by Western blotting. D, cortactin tyrosine phosphorylation is required for efficient HNSCC migration. UMSCC2 cells were assayed for motility by electric substrate impedance sensing in the presence of 10 ng/mL EGF for 24 hours following transfection with control siRNA conjugated to FITC (FITC Control), cortactin siRNA (siRNA), murine FLAG–wild-type cortactin (Cortactin o/x), murine FLAG–wild-type cortactin with cortactin siRNA (Cortactin o/x + siRNA), and murine FLAG–TPM cortactin with cortactin siRNA (Cortactin TPM + siRNA). Columns, percentage motility compared with FITC control-transfected siRNA cells (100%); bars, SE for three independent experiments. *, P < 0.05 compared with FITC control cells.
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