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ORIGINAL ARTICLE

Overexpression of cortactin in head and neck squamous cell carcinomas can be uncoupled from augmented EGF receptor expression

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Abstract

Background. The gene encoding cortactin, *CTTN* (locus 11q13), an actin-binding substrate of Src kinases, is frequently amplified in breast and head and neck squamous cell carcinomas (HNSCC) and cortactin overexpression is thought to contribute in a significant way to the invasive phenotype of these tumors. Elevated Epidermal Growth Factor receptor (EGFR) expression is also commonly observed in HNSCC and has been associated with poor prognosis and resistance to cytotoxic agents, including ionizing radiation. It has been suggested that cortactin overexpression may increase EGFR levels in these tumors by affecting receptor downregulation, however we recently found by multivariate analysis, that cortactin expression status remained an independent prognostic factor for local recurrence, disease-free survival, and overall survival. *Material and Methods.* To examine the potential link between cortactin overexpression and EGFR status, we compared cortactin and EGFR levels in a series of tumor lines derived from HNSCC. RNAi-mediated silencing was performed in cortactin overexpressing cells and *in vivo* tumoral potential with respect to cortactin and EGFR status was analyzed. *Results and Discussion.* Cortactin and EGFR levels were not strictly coupled in these lines and cortactin depletion did not decrease steady state receptor levels, although it did affect the epithelial to mesenchymal phenotypic conversion of cells. These results, together with clinical findings point to the existence of an EGFR-independent role of cortactin in HNSCC that may have important implications regarding the design of targeted therapies to combat tumor spread.

The molecular links that bridge extracellular matrix receptors to the motility machinery of invasive cells have yet to be fully understood. Cortactin, a cortical actin binding protein, as its name indicates, represents a good candidate. Originally identified by the group of Parsons as a substrate of the Src oncogene [1], cortactin was subsequently found to be encoded by a gene frequently amplified in human tumors [2]. It is localized in dynamic actin assembly sites (such as lamellipodia, endosomes, podosomes and invadopodia) and has been implicated in cell motility and invasion, endocytosis and membrane trafficking and in transducing transmembrane signaling to the cytoskeleton (reviewed in [3]).

Cortactin is encoded by the *CTTN* gene (formerly *EMS1*), localized on chromosome locus 11q13. Gene amplification, which occurs in approximately 15% of human breast cancers and over 30% of head and neck cancers, correlates with lymph node metastasis and poor clinical outcome [4–6]. Our recent studies of cortactin expression in squamous cell carcinomas of the head and neck (HNSCC) revealed that increased levels of the protein are associated with significantly increased local recurrence rates, decreased disease-free survival and decreased 5-year overall survival [7]. The major cause of death from squamous cell carcinomas of the head and neck (HNSCC) is local recurrence and

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spread to other organs. A key role for cortactin in tumor invasion and metastatic spread is supported by cortactin overexpression studies in cultured cells and in animal models, and by loss-of-function analyses in cortactin-overexpressing tumor lines.

Cortactin is a multidomain adaptor protein that engages in several protein-protein interactions that may be functionally relevant for tumoral progression. It contains an N-terminal acidic domain that binds to the actin-regulatory Arp2/3 complex, an actin-binding region composed of 6 and one-half, 37-amino acid tandem repeats, a helical region, a proline-rich domain containing Src phosphorylation sites and a C-terminal Src Homology (SH3) domain. A growing list of cortactin partners have been found to associate with the C-terminal SH3 domain including proteins that control actin polymerization and contractility (N-WASP, WIP and EC-MLCK), proteins that regulate receptor clustering, endocytosis and vesicle transport (CortBP1/Shank2, CD2AP CIN85 related protein, dynamin 2) and GTPase regulators (Fgd1, the Cdc42 GEF, BPGAP1 a RhoGAP and the Arf6 effector AMAP1).

Regulation of membrane receptor endocytosis and recycling by cortactin has been proposed to play a role in the progression of tumors with elevated Epidermal Growth Factor receptor (EGFR) levels. Interestingly, in cells derived from head and neck carcinomas, in which overexpression of the EGFR has been associated with poor prognosis [8,9], cortactin overexpression was found to inhibit ligand-dependent EGFR down-regulation and enhance signaling [10]. However, in our analysis of cortactin and EGFR expression in a set of 176 head and neck tumors, rather than observing a strict linkage between cortactin and EGFR overexpression, we identified a subset of cortactin-overexpressing patients (36%) with low EGFR levels [7]. The poor survival rate of these patients equalled that of patients with tumoral overexpression of both EGFR and cortactin. To better understand the contribution of cortactin overexpression to the control of EGFR levels and the tumoral phenotype in head and neck carcinomas, we analyzed cortactin levels and performed loss of function studies in cultured HNSCC lines in which EGFR levels and tumorigenic potential had been previously well characterized [8,11,12]. In these lines cortactin and EGFR expression were not coordinately regulated. Cortactin overexpressing cells with moderate EGFR levels and EGFR overexpressing cells with moderate cortactin levels both gave rise to rapidly growing tumors with distinct phenotypes and divergent sensitivities to therapeutic agents.

Material and methods

Cell cultures

Three human head and neck cancer cell lines (CAL33, CAL60 and Detroit 562) and two breast cancer lines (CAL51 and MCF-7) were used. CAL33, CAL60 and CAL51 lines were established in the Antoine Lacassagne Cancer Centre. Detroit 562 cells derived from a metastatic pharyngeal SCC were from American Type Culture Collection (Rockville, MD, USA). All cells, including the normal human embryonic kidney line HEK293, were cultivated in DMEM (Invitrogen, Cergy Pontoise, France) containing penicillin (50 U/ml), streptomycin (50 μg/ml) and 8% (v/v) fetal calf serum (FCS), or 10% FCS for Detroit 562 cells.

Antibodies

Mouse monoclonal anti-cortactin (clone AF11) and rabbit anti-phospho-cortactin (Tyr 421) polyclonal anti-body were from Upstate (Chemicon International, Ltd., Hampshire, UK), mouse monoclonal anti-EGFR (Ab-12 cocktail R19/48) was from Thermo Scientific (Interchim, Montlucon, France) and rabbit polyclonal anti-cortactin (H-191) antibody was from Santa Cruz Biotechnology, Inc. (Tebu-Bio SA, Le Perray en Yvelines, France). Monoclonal antiEGFR (clone 31G7) was from Invitrogen-Zymed Laboratoires. The rabbit polyclonal anti-ERK1/2 generated in the laboratory was used as control. Secondary antibodies coupled to horseradish peroxidase or alkaline phosphatase were from Promega France (Charbonnières-les-Bains, France) and New England BioLabs (Beverly, MA), respectively. Biotinylated swine polyclonal anti-rabbit immunoglobins was purchased from Dako France (Trappes, France). Fluorescently labelled secondary antibodies for immunofluorescence were from Molecular Probes (Eugene, OR).

Western blot analysis

Exponentially growing cells at 80–90% confluence were gently washed twice in PBS at room temperature, scraped in 4X Laemmli buffer. Resulting cell lysates were sonicated, subjected to SDS-PAGE on 7.5% acrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P Transfer Membrane, Guyancourt, France). Immune complexes on membranes were detected by enhanced chemiluminescence using horseradish-peroxidase- or alkaline-phosphatase-based substrates from Pierce (Rockford, IL) and New England BioLabs, respectively. Where indicated, band intensity was quantified

using the GeneGnome chemiluminescent imaging system (Syngene, Frederick, MD).

Southern blot analysis

Total genomic DNA was prepared by proteinase K digestion and isopropanol extraction. For each cell line, 20 μg of genomic DNA was digested with appropriate restriction enzymes (HindIII, EcoRI, XhoI) and separated by electrophoresis on 1.5% agarose gel. Total DNA was transferred to nylon membranes (Hybond-N, Amersham-Buchler, Braunschweig, Germany) and fixed by UV light. The membranes were prehybridized at 60°C for 2 hours in Church Buffer (1% BSA, SDS 20%, NaH_2PO_4 0.5M, pH 7, EDTA 0.5M) and then hybridized in the same buffer at 60°C over night with a radio-labeled 373bp PCR fragment of the cortactin gene (sense primer: 5′-CAGGAGCAGATGCACCACTA-3′; antisense primer: 5′-GTTTCGAGCTCCCTGTCAAG-3′). After hybridization the blots were washed and autoradiography was performed.

Northern blot analysis

For each cell line, 20 μg of total RNA was separated by electrophoresis on 1% agarose gel, containing formaldehyde 37% and MOPS 1X (N-morpholino propanesulphonic acid). Then RNA was transferred to nylon membranes (Hybond-N, Amersham-Buchler, Braunschweig, Germany) and fixed with UV light. The membranes were prehybridized at 60°C Church Buffer then hybridized in the same buffer at 60°C overnight with a radio-labeled probe corresponding to a 341bp fragment in the coding region of the human cortactin mRNA (sense primer: 5′-CCTATGCCACAGAGGCTGTC-3′; antisense primer: 5′-CCACCCAAGAACCAAAGAAG-3′). As control, a 157bp fragment corresponding to the human acid ribosomal phosphoprotein PO (36B4) was used (sense primer: 5′-GATTGGCTACCCAACTGTTG-3′; antisense primer: 5′-CAGGGGCAGCAGCCACAAAG-3′). Probes were labeled using the RediPrime DNA labelling system (Invitrogen). Autoradiographic signals were quantified by scanning densitometry.

RNA interference

For stable transfection, Detroit 562 cells were grown to 90–95% confluence before transfection using LipofectamineTM 2000 (Invitrogen Corporation) with the pTER vector containing an shRNA sequence targeting the human cortactin transcript (Operon 5′-CAUAUCAACAUACACAAGCdTdT-3′). Following transfection, cells were incubated at 37°C in DMEM for 24 hours before adding Zeocin (250mg/ml). After 3 weeks, clones expressing the pTER-cortactin shRNA were isolated and amplified for analysis. Transient transfections were carried out with the indicated plasmid using the Amaxa Nucleofactor Kit V (Amaxa GmbH, Cologne, Germany) according to the manufacturer's instructions.

Immunofluoresence

For immunostaining, cells were seeded on gelatin-coated glass coverslips in 12-well plates. After 24 hours, they were fixed (3% paraformaldehyde/2% sucrose) and permeabilized with 0.2% Triton-X100 for 5 min at room temperature prior to staining, image acquisition and analysis, as described in [13].

Immunohistochemistry

Staining was performed on paraffin embedded sections (4 μM) of tumors generated in nude mice following injection of HNSCC tumor lines Detroit 562, CAL60 and CAL33. Tumor blocks were stained with haematoxylin & eosine to assess differentiation and proliferation of the tumor, and the most informative blocks with the largest tumor/stroma interface were selected for immunostaining. Cortactin labeling was performed with rabbit polyclonal anti-cortactin antibody (1:250 dilution) on de-waxed sections after heat-mediated antigen retrieval using a Bench Mark XT slide preparation system (Ventana Medical Systems, Inc.), followed by a counterstaining in Mayer's hematoxylin. EGFR staining with rabbit polyclonal anti-EGFR antibody (1:10 dilution) was performed manually on sections following proteolytic epitope retrieval with proteinase K (Dako, France S.A.S., Trappes, France) using and the ARK™ (Animal Research Kit) Peroxidase (Dako). Stained sections were observed with a Leica DMR microscope equipped with a CoolSNAP EZ camera (Roper Scientifique, Evry, France).

Tumor formation in nude mice

Aliquots of 15×10^6 cells were frozen in FCS supplemented with 5% DMSO (vol/vol) prior to injection into mice. Shortly before injection, cells were thawed and resuspended in Ringer lactate. Animal experiments were performed in accordance with the regulations of the institutional ethical commission and of the United Kingdom Coordinating Committee on Cancer Research guidelines (as reported in [11]. Six-week-old female Swiss nude mice were purchased from Charles River (L'Arbresle, France) and received subcutaneous inoculation of 2×10^6 cells dissolved in 100 μl of Ringer lactate in the right flank. Six to eight animals were used per treatment condition. Tumor length and width were measured weekly using a

caliper and tumor volume was calculated as $\pi 6 \times$ length $\times$ width^2 until animals were sacrificed by spinal cord dislocation. Tumors were surgically removed and weighed. Half of each tumor was frozen in liquid nitrogen immediately for protein analysis and the other half was fixed in paraformaldehyde overnight then embedded in paraffin for immunohistochemical examination.

Results

CTTN gene amplification and mRNA overexpression in HNSCC lines

To study the consequences of deregulated cortactin expression on EGFR levels and tumoral potential, we first analyzed selected HNSCC cell lines with respect to *CTTN* gene amplification and expression of the protein. These lines, established in our Institute or available from the ATCC, had previously been characterized for EGFR levels [8], doubling time, *in cellulo* sensitivities to gefitinib or irradiation and p53 status, as reported in [11]. The status of *CTTN* gene amplification was determined by Southern blotting, as shown in Figure 1 (top). Detroit 562 cells, with known amplification [14], displayed a 2.5-fold greater hybridization intensity relative to DNA isolated from MCF-7 cells, a well characterized breast cancer line also known to display increased *CTTN* gene copy number. In contrast, no amplification was observed in CAL60 and CAL33 HNSCC lines, CAL51 breast cancer cells or HEK293 human embryonic kidney cells, used as control. A transcript of approximately 3.4Kb corresponding to the full length cortactin mRNA was detected in all lines examined by Northern blot analysis (Figure 1, bottom). Consistent with the observed gene amplification, significant overexpression of this mRNA was observed in the Detroit 562 line (5.4-fold, relative to HEK293 cells). Cortactin transcript levels were also elevated, with respect to HEK293 cells, in the other HNSCC lines (3.0- and 3.5-fold in CAL33 and CAL60 cells, respectively) and in the breast cancer lines (3.1-fold and 2.9-fold in CAL51 and MCF-7, respectively). In addition to the 3.4Kb transcript, a smaller mRNA species of approximately 2.6Kb was detected in the HNSCC lines and MCF-7 cells. This faster migrating mRNA, most abundant in Detroit 562 and CAL60 HNSCC cells, was observed in Northern blot analyses using two different PCR-generated probes and likely corresponds to a cortactin splice variant lacking approximately 800bp in the 3′ untranslated region (*e.g.* GeneBank Accession BC033889.1).

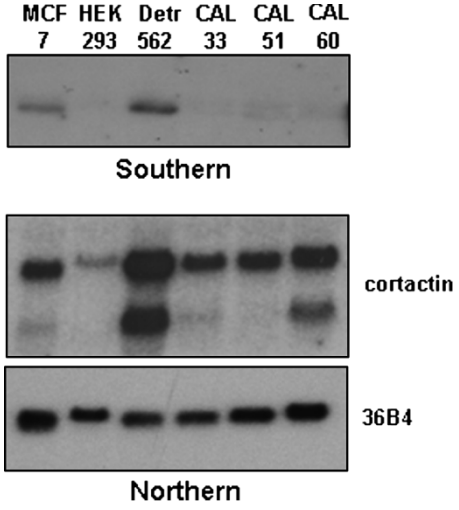

Figure 1. *(top)*. *CTTN* amplification and cortactin mRNA expression in human HNSCC lines (Detroit 562, CAL33, CAL60 and CAL33), breast tumor lines (MCF-7 and CAL51) and HEK293 human embryonic kidney cells. The Southern blot was hybridized with a cortactin cDNA probe and autoradiographed over night. Similar results were obtained in 2 independent experiments. *(bottom)* The Northern blot was sequentially hybridized with probes for cortactin and 36B4, as loading control.

P80/85 cortactin overexpression

Western analysis of cortactin expression is shown in Figure 2. The protein was detected as a p80/85 doublet in all cell lines examined using both the monoclonal 4F11 antibody that recognizes an epitope in the 5th tandem repeat region of the protein [15], and a polyclonal antibody directed against the C-terminal 196 residues of the protein (i.e. distal to the actin-binding tandem repeat region (not shown). In all cases, the lower band (p80) was more intense than the upper band (p85). Among the HNSCC lines examined, Detroit 562 cells expressed the highest levels of cortactin (6-fold relative to HEK293 cells), followed by CAL60 and CAL33 (5-fold and 3-fold, respectively). Expression in the breast cancer lines (MCF-7 and CAL51) is approximately 3-fold higher than in HEK293 cells. Thus,

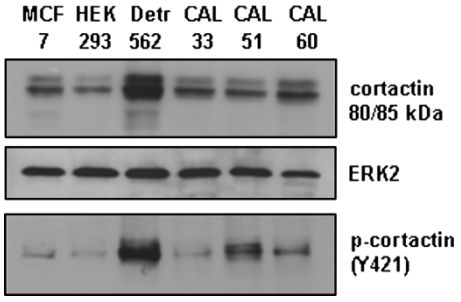

Figure 2. Western blot analysis of cortactin expression and phosphorylation. Total p80/p85 cortactin expression in the indicated cell lines was determined using the monoclonal 4F11 antibody and phosphorylation on residue 421 was detected with a rat polyclonal antibody.

Detroit 562 was the highest cortactin overexpressing line relative to the other two HNSCC cell lines, CAL60 and CAL33, with intermediate cortactin expression levels.

In the HNSCC lines, phosphorylation of cortactin on tyrosine residue 421, previously reported to be required for efficient cell movement [16], was also highest in Detroit 562 cells, and followed by CAL60 and CAL33 cells (Figure 2, bottom).

EGFR expression in HNSCC lines

Similar to cortactin gene amplification, EGR receptor overexpression in head and neck tumors have been reported to correlate with an aggressive phenotype and unfavorable clinical outcome [8,17] for review). A casual link between these events (cortactin gene amplification and EGFR overexpression) in HNSCC has been proposed, in light of data indicating that cortactin plays a role in regulating ligand-induced EGFR down-regulation [10,18]. Therefore, we examined EGFR expression in the different cell lines by Western blotting. The highest levels of the protein were observed in CAL33 and CAL60 cells, and the lowest level in Detroit 562 cells (Figure 3). These results are consistent with EGF binding activity, previously reported for these lines [11]. Hence, EGFR expression in the HNSCC lines did not correlate with cortactin expression. Receptor expression was undetectable by Western analysis in CAL51 breast cancer cells, despite cortactin levels that equaled those of CAL60 and CAL33 cells. For further studies, we focused our attention on the three HNSCC lines and with high (Detroit 562 cells) or intermediate (CAL60, CAL33) levels of cortactin expression.

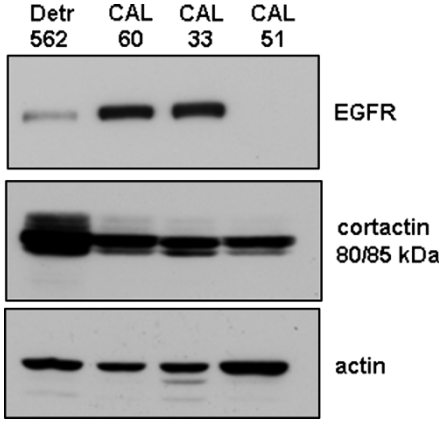

Figure 3. Western blot analysis of EGFR (EGFR) and cortactin expression in total lysates from the indicated cell lines is shown. ERK2 expression was determined as loading control. Representative results from three independent analyses are shown.

Cortactin silencing in Detroit 562 cells

To examine the regulation of EGFR expression by cortactin in the 3 HNSCC lines we performed RNA silencing by transient transfection of a pTER-shRNA construct targeting cortactin mRNA. As shown in Figure 4, cortactin expression was significantly decreased following transfection of Detroit 562, CAL 60 and CAL33 to 26, 14 and 7% of control levels, respectively. In each case, we failed to detect a decrease in EGFR expression after cortactin depletion, suggesting that cortactin does not down-regulate steady state EGFR levels.

In addition to EGFR modulation, cortactin has been shown to regulate several cell functions that could contribute to the invasive phenotype of HNSCC when overexpressed, including actin reorganization in cortical networks and invadopodia, receptor-mediated endocytosis and vesicle trafficking (reviewed in [3]. To examine the morphotypic consequences of cortactin depletion in HNSCC, we isolated stable clones Detroit 562 cell clones following transfection with the pTER cortactin plasmid or empty vector. As shown in Figure 5, clones with varying degrees of silencing were obtained, including clones with up to $>90\%$ reduction in cortactin expression. Surprisingly, immunofluorescent staining revealed that the zeocine-resistant clones consisted of mosaic populations containing cortactin-depleted and cortactin-overexpressing cells. This result is illustrated by staining of the clone AE, shown in Figure 6 (right panel). With time in culture, expression of the cortactin-targeting shRNA was progressively lost and the number of cortactin-expressing cells increased. Despite multiple attempts to establish a cortactin-depleted cell line, we were unable to maintain stable expression of the targeting shRNA following prolonged culture of selected clones. Interestingly however, the cortactin-overexpressing cells in

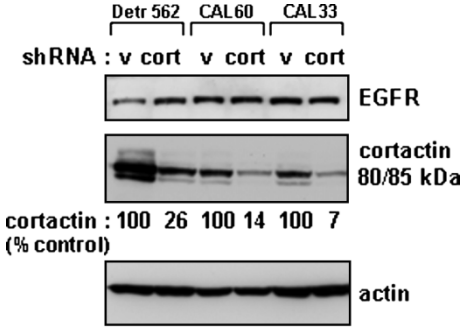

Figure 4. Cortactin silencing in Detroit 562 cells. Cortactin expression levels determined by Western analysis of 10 μg total cell lysates 48 hours following transfection of the indicated cell lines with an empty pTER vector (v) or a cortactin targeting vector. Quantification of cortactin silencing (% cortactin expression relative to control) is indicated below. ERK2 was determined as protein loading control.

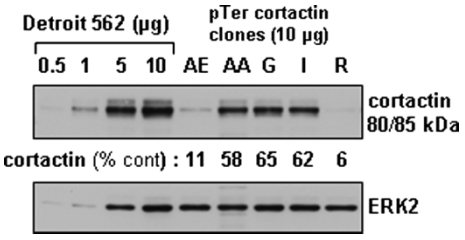

Figure 5. Cortactin silencing in Detroit 562 cells. Cortactin expression levels determined by Western analysis of 10 µg total cell lysates from stable pTER-cortactin transfectants is compared to expression of the protein in 0.5–10 µg of parental cell lysate. Quantification (% 10 µg of control lysate), performed on a shorter exposure of the film, is indicated below. ERK2 was determined as protein loading control.

the zeocine-resistant populations displayed a stellate morphology, characteristic of tumor epithelial cells that had undergone a mesenchymal transition, and their localization was enriched in the periphery of epithelial islands formed by cortactin-depleted cells.

Cortactin expression and tumoral progression

In parallel, we investigated the status of cortactin expression with relation to the tumorigenic potential and EGFR levels in nude mice of the 3 HNSCC lines (Detroit 562, CAL60 and CAL33) for which levels of cortactin and EGFR expression in culture had been determined. Subcutaneous injection in nude mice of Detroit 562 cells induced rapidly growing tumors comprised of small, poorly differentiated cells. Whereas staining with anti-cortactin antibody was absent in normal mouse skin (not shown), cortactin staining in Detroit 562-derived tumors was intense (Figure 7A), consistent with *CTTN* gene amplification in this line. All tumor cells displayed cytoplasmic cortactin staining and membrane reactivity was detected in protrusive structures of tumor cells at the invasive front of the carcinoma (Figure 7A, inset). In line with findings in cultured cells, EGFR levels in Detroit 562 tumors remained low (Figure 7B), suggesting that cortactin overexpression in an *in vivo* setting did not increase EGFR expression.

In the case of CAL60 cells, that express moderate levels of cortactin in culture, tumor growth in mice was intermediate. Injection of these cells gave rise to well differentiated tumors with intermediate levels of cortactin staining that localized to cell-cell junctions in more highly differentiated cells (Figure 7A and Table I). EGFR expression in tumors from these cells was most intense in the least differentiated basal layers (Figure 7B and Table I). CAL33 cells, that display the highest EGFR binding activity in culture [11] and the weakest expression of cortactin, gave rise to rapidly growing tumors with moderate cortactin immunoreactivity (Figure 7A). As shown in Figure 7B, EGFR staining in xenographs of these cells was intense. Thus, both our *in celulo* and *in vivo* studies on HNSCC lines with different cortactin and EGFR expression profiles suggest that cortactin overexpression may not be functionally coupled to EGFR overexpression.

Other tumoral properties of these HNSCC lines *in vivo*, including Vascular Endothelial Growth Factor levels and response to treatment with an anti-vascular agent and the EGFR tyrosine kinase inhibitor gefitinib, have been described in [11].

Discussion

Here we have analyzed cortactin expression in selected HNSCC lines, with respect to their, EGFR levels, phenotype in cultured monolayers and tumoral potential. Our main findings are that i) cortactin expression is not strictly linked to EGFR levels in these cells, and ii) both cortactin overexpressing lines (with moderate EGFR levels) and EGF overexpressing lines (with moderate cortactin expression) can give rise to tumors with distinct growth properties and responses to therapeutic agents.

mRNA expression

As compared to control HEK293 epithelial cells, cortactin mRNA levels were elevated in all of the HNSCC and breast tumor lines examined, even in absence of gene amplification. This indicates that cortactin expression can be controlled at multiple levels and increased transcription is likely to be downstream of activated autocrine signaling pathways in these tumor lines. Alternative splicing is another important mechanism for regulating gene expression and protein function. Increasing evidence suggests that aberrant splicing may play a role in cancer [19]. In addition to the full length cortactin mRNA of 3.4, a transcript of 2.6kb was expressed to varying degrees in all the HNSCC cells, with highest levels in Detroit 562 and CAL 60 cells. As indicated above, this most likely corresponds to an mRNA with an 800bp deletion in the 3′ untranslated region of the transcript that, if translated, would not affect the protein sequence yet may affect its expression.

Probing cortactin function

Thus far, total cortactin knock-out models have not yet been successfully generated due to premature differentiation of embryonic stem cells with a deletion of one allele (personal communication in [20]).

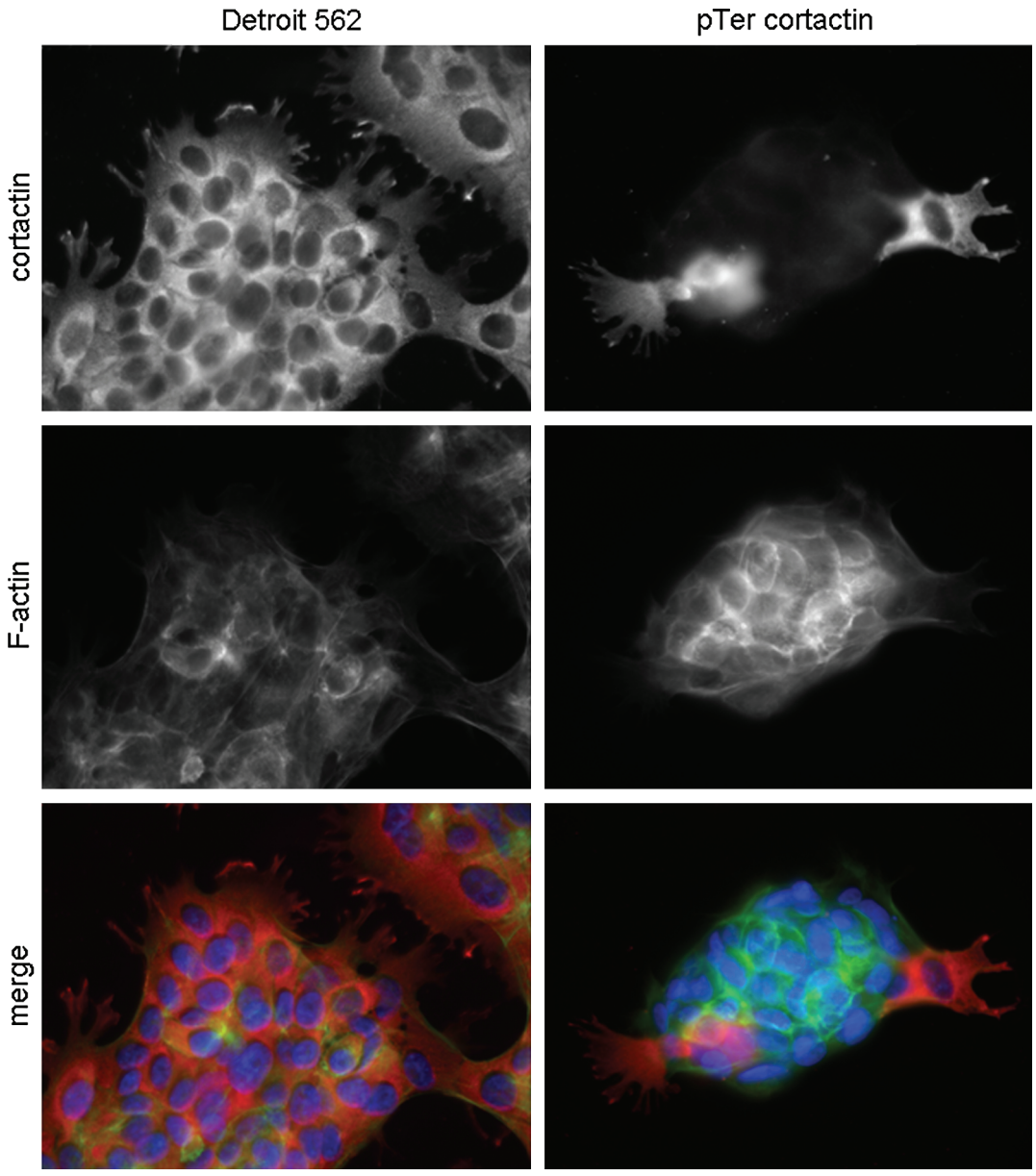

Figure 6. Cortactin silencing induces morphological changes in Detroit 562 HNSCC cells. Detroit 562 parental cells and pTER-cortactin-transfected cells (clone AE) were fixed, permeabilized and co-stained, as indicated, for cortactin (H-191) and F-actin (Alexa 488-phalloidin) and DAPI. Alexa 546-conjugated anti-mouse secondary antibody was used. Merge images show co-localization of cortactin (red), F-actin (green) and DAPI (blue) (63X/1.4 NA oil objective; scale bar, 20μm).

However, loss-of-function studies using siRNA approaches in different cellular systems have provided valuable information concerning the role of cortactin in various processes including actin remodelling and invasion, endocytosis, regulation of intercellular adhesion and pathogen infection. Cortactin silencing in Detroit 562 cells represents a useful *in cellulo* model to study mechanisms involved in epithelial-mesenchymal transition and the promotion of invasive behavior by the protein. At present we can not explain the loss of shRNA expression observed in selected clones (DNA rearrangement, epigenetic silencing, *etc.*) and we are undertaking different strategies to circumvent this problem.

Importantly for our studies, cortactin silencing in the Detroit 562 line was found to regulate EGFR levels by inhibiting ligand-stimulated receptor down-regulation [10]. In our attempts to extend these observations, we were unable to detect a significant decrease in EGFR levels following cortactin silencing. It is noteworthy that in our experiments, we determined steady state EGFR levels in growing cells, as opposed to measuring receptor levels following acute ligand stimulation, as reported by Timpson et al.

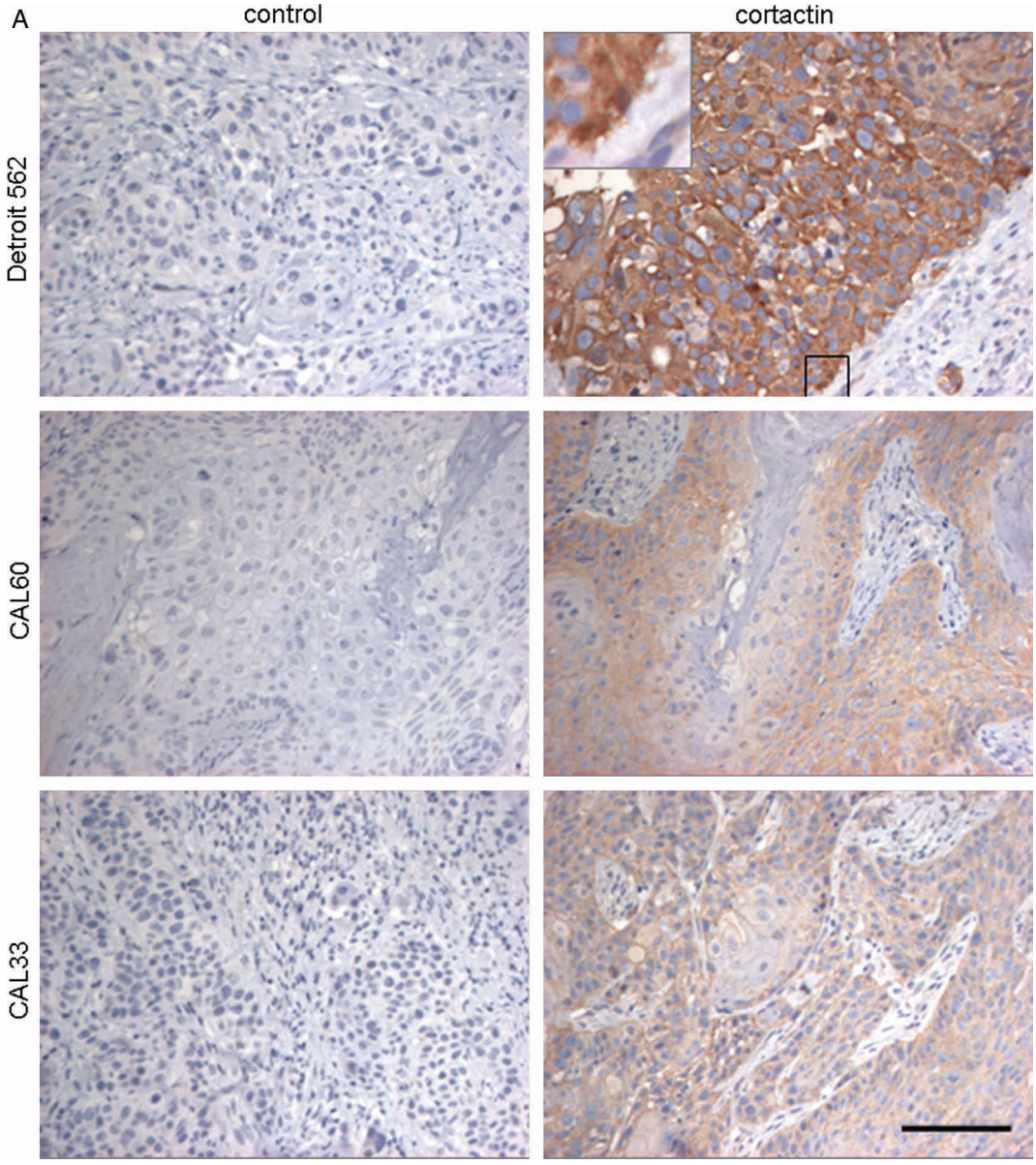

Figure 7 (*Continued*)

[10]. Nonetheless, in the HNSCC lines examined here, EGFR expression levels were not strictly related to cortactin levels. Hence, Detroit 562 cells with the highest cortactin expression displayed the lowest EGFR expression whereas CAL33 with extensive EGFR overexpression had lower cortactin levels, both in culture and *in vivo*.

Cortactin and the tumoral phenotype

The mouse tumor model reported here provides useful information about the biological properties of different HNSCC xenographs and provides a platform for moving to *in vivo* experiments using an orthotopic tumor model. We confirmed that cortactin and EGFR expression by the tumor cells *in vivo* faithfully reflected the pattern observed in cultured cells. Interestingly, both Detroit 562 and CAL33 cells gave rise to rapidly growing tumors with marked disparity in their angiogenic potential (tumor Vascular Endothelial Growth Factor levels) and response to treatment with the microtubule-disrupting anti-vascular agent agent ZD6126 [11]. Detroit 562-induced tumors with high cortactin levels displayed low levels of Vascular Endothelial Growth Factor. Further, these tumors were particularly resistant to ZD6126. Surprisingly, it was found that microtubule disruption by this compound actually enhanced tumoral growth of cortactin-overexpressing Detroit 562 cells. In contrast, the CAL33

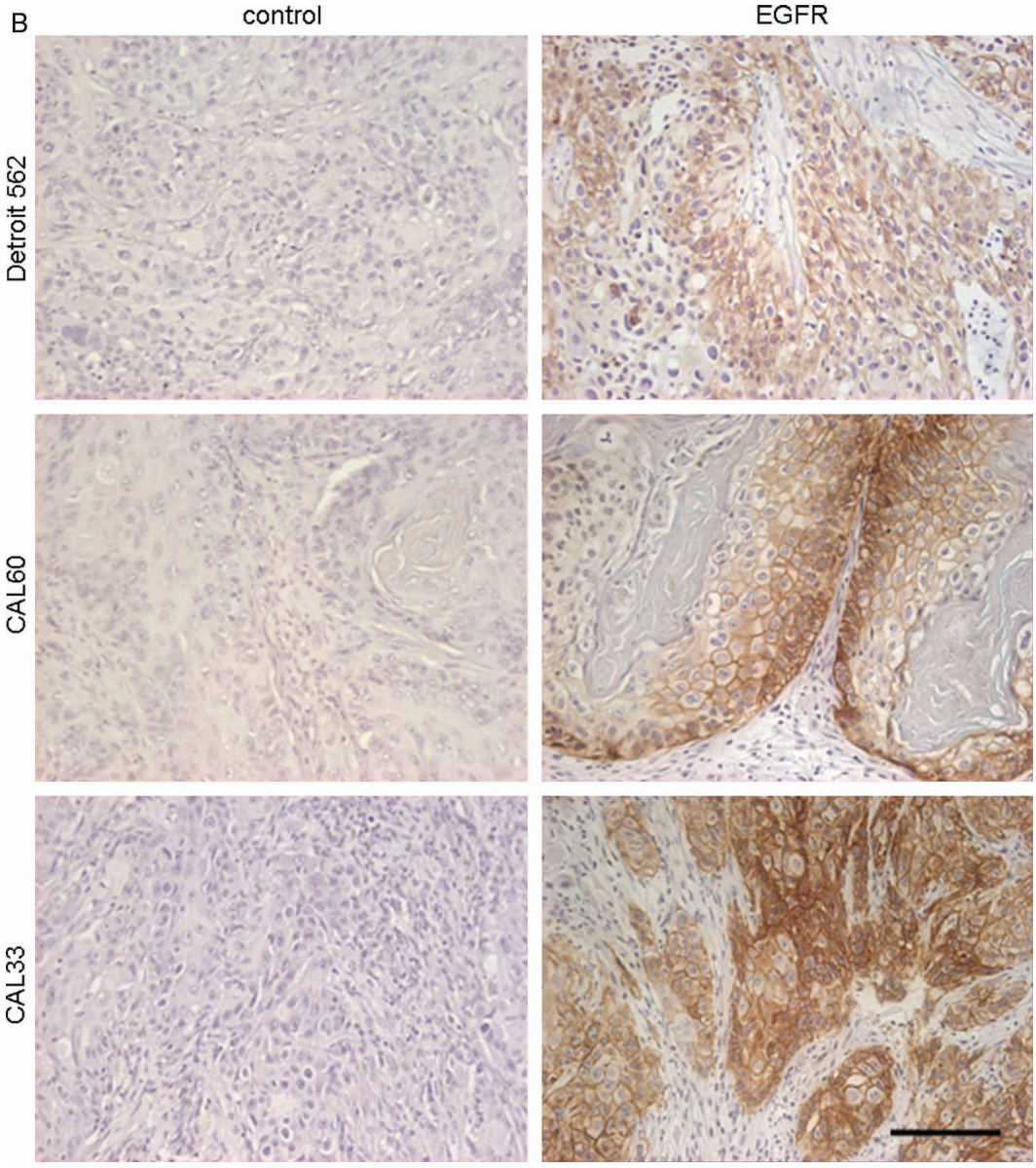

Figure 7. Cortactin and EGFR expression in xenographs from HNSCC lines Detroit 562 (top), CAL60 (middle) and CAL33 (bottom). A. Immunohistochemistry was performed on paraffin-embedded material using rabbit polyclonal anti-cortactin antibody (cortactin), or in absence of primary antibody (control), as described in Material and Methods. Area shown in inset is indicated. B. EGFR staining was performed with polyclonal anti-EGFR antibody (EGFR); non-specific staining in absence of primary antibody (control) is shown. Scale bars in A and B = 100μm.

xenographs with high EGFR and low cortactin expression displayed high tumor Vascular Endothelial Growth Factor levels and sensitivity to the anti-vascular drug in combination with an EGFR kinase inhibitor. It remains to be determined whether a casual link exists between cortactin overexpression and response to this drug.

Therapies that target the EGFR have generated high hopes for the management of head and neck cancer. Cortactin is generally accepted as a downstream target of the EGFR kinase/Src kinas axis in HNSCC. Accordingly, Rothschild et al. have recently shown that serum-stimulated tyrosine phosphorylation of cortactin is inhibited by the EGFR inhibitor gefitinib (Iressa/ ZD1839) in a panel of HNSCC lines [21]. Importantly, in these lines which reportedly expressed equivalent EGFR levels, gefitinib-inhibited cell migration in response to EGF and serum was inversely proportional to the level of cortactin expression following cortactin-targeted siRNA knockdown [21]. Consistent with this observation, Magne et al. established using an MTT assay that the Detroit 562 line was less sensitive to gefitinib than CAL 60 and CAL33 cells [12]. In this latter study, drug sensitivity was correlated with EGFR levels, rather than cortactin levels.

Table I. *In vivo* growth and expression of cortactin and EGFR.

Parameters Evaluated	Detroit 562	CAL60	CAL33
Tumor growth	rapid	intermediate	rapid
Tumor cortactin staining	high	intermediate	intermediate
Tumor EGFR staining	intermediate	intermediate	high
IHC observations	small, poorly differentiated	well differentiated	junctional staining in differentiated cells

Quantitative results of tumor growth analyses, response to treatment and VEGF levels, have been reported in [31].

Counterintuitively, EGFR expression has not proven to be strong predictive biomarker for response to EGFR targeting agents in the clinic. Thus, the presence of amplified downstream signalling (*e.g.* cortactin overexpression) or cooperative signalling by multiple kinases may play an important role both in driving tumoral growth and providing salvage pathways for cells to resist EGFR antagonists. Thus, in a given cellular context the acquisition of a motile phenotype by cortactin overexpression may bypass the requirement for elevated EGFR signalling, especially in the case of amplification of other genes in the 11q13 locus, including CCND1 (which is amplified in Detroit 562 cells). Indeed, in a recent tissue microarray analysis of cortactin and EGFR expression in head and neck carcinomas we identified a subset of cortactin overexpressing tumors, without EGFR overexpression, that displayed increased tumoral recurrence and decreased overall survival rates that were comparable with tumors that overexpress both cortactin and the EGFR [7]. Thus, cortactin status may be an important parameter to consider for the development of therapeutic strategies (*e.g.* Src kinase inhibitors, cortactin SH3-directed compounds) to combat head and neck cancer.

While this manuscript was in preparation, Timpson et al. reported that increased cortactin expression may enhance signalling by the c-MET receptor tyrosine kinase, likely by attenuating ligand-induced down-regulation [22]. This finding is important in light of the increased expression of this receptor in HNSCC [23] and recent reports indicating that c-MET amplification/activation may contribute to gefitinib resistance by providing an independent input to common signalling networks. Further elucidation of cortacin function should help to understand the molecular pathogenesis of HNSCC.

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