Clin Exp Metastasis (2008) 25:685–693
DOI 10.1007/s10585-007-9121-7

RESEARCH PAPER

Bypassing cellular EGF receptor dependence through epithelial-to-mesenchymal-like transitions

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Received: 18 July 2007 / Accepted: 16 October 2007 / Published online: 31 January 2008

Abstract Over 90% of all cancers are carcinomas, malignancies derived from cells of epithelial origin. As carcinomas progress, these tumors may lose epithelial morphology and acquire mesenchymal characteristics which contribute to metastatic potential. An epithelial-to-mesenchymal transition (EMT) similar to the process critical for embryonic development is thought to be an important mechanism for promoting cancer invasion and metastasis. Epithelial-to-mesenchymal transitions have been induced in vitro by transient or unregulated activation of receptor tyrosine kinase signaling pathways, oncogene signaling and disruption of homotypic cell adhesion. These cellular models attempt to mimic the complexity of human carcinomas which respond to autocrine and paracrine signals from both the tumor and its microenvironment. Activation of the epidermal growth factor receptor (EGFR) has been implicated in the neoplastic transformation of solid tumors and overexpression of EGFR has been shown to correlate with poor survival. Notably, epithelial tumor cells have been shown to be significantly more sensitive to EGFR inhibitors than tumor cells which have undergone an EMT-like transition and acquired mesenchymal characteristics, including non-small cell lung (NSCLC), head and neck (HN), bladder, colorectal, pancreas and breast carcinomas. EGFR blockade has also been shown to inhibit cellular migration, suggesting a role for EGFR inhibitors in the control of metastasis. The interaction between EGFR and the multiple signaling nodes which regulate EMT suggest that the combination of an EGFR inhibitor and other molecular targeted agents may offer a novel approach to controlling metastasis.

Keywords Epithelial-to-mesenchymal transition · EMT · EGFR · IGF-1R · PDGFR · Cancer · Metastasis · Erlotinib · Snail · Zeb-1 · Twist · Vimentin · E-cadherin

Introduction

Human cancers rely on multiple overlapping signal transduction pathways to activate and regulate cellular proliferation, survival and migration programs. The epithelial-to-mesenchymal transition (EMT) is a critical process in embryonic development for metazoan organisms and a similar process has also been shown to play a role in oncogenic progression and metastasis. Tumor metastasis involves a sequential series of processes which promote and regulate the escape of migratory cancer cells to generate metastatic lesions at distant sites. The process begins in the primary tumor, where tumor cells dysregulate homotypic cell adhesion, downregulate cell adhesion proteins such as E-cadherin, and upregulate proteins characteristic of a more motile, mesenchymal-like phenotype such as vimentin. This process requires transcriptional reprogramming to suppress E-cadherin expression via transcription factors associated with EMT (for review see [1]). Tumor cells undergoing EMT have been shown to undergo "cadherin switching", downregulating E-cadherin and compensating with alternate cadherin proteins such as N cadherin [2]. There is evidence that the downregulation of E-cadherin and upregulation of proteins characteristic of a mesenchymal phenotype may occur preferentially at the

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invasive edge of a tumor [3]. Initiation of metastasis requires this initial disruption of cell–cell junctions and gain of cellular motility, permitting individual cells to migrate away from the primary tumor. In order to migrate through the surrounding extracellular matrix (ECM) cells may upregulate secreted proteases such as the matrix metalloproteinases (MMPs). These motile, invasive cells may then cross the endothelial cell barrier and intravasate into the bloodstream. Once in the bloodstream, these mesenchymal-like cancer cells can travel to distant sites where they extravasate the endothelial cell wall to colonize in a new, supportive niche. Primary tumor cells of specific cellular origins have been shown to preferentially colonize specific tissues, although the reasons for this are not entirely clear. However it is commonly accepted that once a metastatic tumor cell has implanted in a niche supportive of proliferation, that cell may undergo a mesenchymal-to-epithelial transition (MET). Consistent with this, the emerging metastatic tumor often resembles the primary tumor from which it derived both in cellular phenotype and multi-cellular architecture. It is not clear whether EMT-like changes are required for all steps in metastasis, and the possibility remains that EMT is a necessary but insufficient step in cancer metastasis.

Cellular biomarkers of EMT

A hallmark of EMT is loss of E-cadherin, a key mediator of cell–cell junctions. Numerous studies have shown a high correlation between loss of E-cadherin, the gain of vimentin and tumor invasiveness in cancer cells and patient tumors (e.g. [4–6]). A down regulation of E-cadherin most frequently results from transcriptional repression, mediated by zinc finger, forkhead domain and bHLH factors including Zeb1/TCF8/δEF1, Zeb2 (Sip1), Snail, Slug, FOXC2 and Twist. The expression of Snail, Slug and specific bHLH transcription factors have been implicated in cell survival and acquired resistance to chemotherapy [7–13]. However loss of E-cadherin alone does not constitute EMT, as cells which harbor a mutation in E-cadherin and have lost functional cell–cell junctions do not acquire the additional morphological and transcriptional changes associated with EMT [14, 15]. These changes include acquisition of cellular markers characteristic of a mesenchymal cell such as vimentin and fibronectin, expression of E-cadherin-repressing transcription factors, and frequently the acquisition of a migratory or "scattering" morphology. Loss of E-cadherin appears to be a prerequisite for tumor progression and not just a consequence of tumor dedifferentiation. In transgenic mice which spontaneously develop pancreatic tumors, E-cadherin expression was shown to decrease with tumor progression, but maintenance of E-cadherin expression during tumorigenesis arrested tumor development at the benign adenoma stage while expression of a dominant negative E-cadherin induced early metastasis [16]. Ectopic expression of E-cadherin is sufficient to suppress tumor cell invasion in vitro and tumor progression in vivo while knock-down of E-cadherin converts cells from non-invasive to invasive [17]. However, ectopic expression of E-cadherin does not restore an epithelial phenotype in cells which overexpress the transcriptional repressor Twist [18]. This implies that restoration of E-cadherin to mesenchymal cells may be insufficient to reverse EMT and confer an epithelial phenotype. Taken together, these observations are consistent with the hypothesis that E-cadherin has a tumor suppressive function and is not simply a marker of tumor differentiation.

Accumulating evidence suggests that EMT occurs at the level of transcriptional reprogramming and chromatin remodeling [18–23]. Several transcription factors have been implicated in the transcriptional repression of E-cadherin, through interaction with specific E-boxes (reviewed in [24]). The zinc finger proteins Snail, Slug, Zeb-1 and Zeb-2 (SIP1) as well as the bHLH factors Twist and E12/E47 have been shown to repress E-cadherin and markers of cell polarity. Ectopic expression of Twist or Zeb-1 is sufficient to downregulate endogenous E-cadherin and induce EMT [18, 20, 25–27]. Snail has been shown to activate the transcription of the mesenchymal biomarkers vimentin and fibronectin. Moreover, expression of Snail has been shown to induce transcriptional downregulation of E-cadherin, to upregulate vimentin, fibronectin and Zeb1 and to promote a fibroblastic, invasive cellular phenotype [28]. The fact that Zeb1 is activated by Snail implies cooperativity between these factors although Zeb1 does not appear to be directly downstream of Snail. Elegant in vivo and in vitro studies using a series of breast carcinoma cell lines with distinct metastatic properties identified Twist as a key regulator of metastasis [18]. Overexpression of Twist in non-cancerous epithelial cells induces expression of mesenchymal cell markers, repression of E-cadherin and an EMT-like phenotype. Consistent with this, suppression of Twist inhibits metastasis in a mouse mammary carcinoma model [18].

Cellular signals promoting EMT-like transitions

Activation of these transcription factors, leading to initiation of EMT and consequently metastasis, may be triggered by a variety of extra- and intracellular signals. One of the first factors observed to induce EMT was "scatter factor" or HGF [29, 30]. Since this early observation, a large number signaling pathways have been shown to induce EMT in vitro. These include growth factors (EGF, VEGF,

TGF-β, Wnt, SDF, PGE2), cytokines (ILEI, interleukins), integrin signaling, extracellular matrix proteins (MMPs), inflammatory signals (COX-2), and potentially stress stimuli such as hypoxia, signaling through non-receptor tyrosine kinases such as Src and oncogenic activation of receptor tyrosine kinases RTKs) [31–35]. These EMT-activating signals can be paracrine, emanating from infiltrating stroma, or autocrine, produced by the tumor cells themselves. One intriguing report suggests that EMT may not only promote the migration of primary breast cancer tumor cells, but may also lead to the formation of non-malignant stromal cells, and this reciprocal interaction may help explain the poor prognosis of some cancers which show evidence of EMT [36].

In addition to effects on proliferation and migration, EMT activators have been shown to promote cell survival through inhibition of apoptosis. Interestingly, TGF-β has been shown to be a potent activator of apoptosis in many cell types including epithelial cells [37, 38]. When treated with TGF-β, most fetal hepatocytes undergo apoptosis, however a fraction survive. Those hepatocytes which have survived TGF-β treatment, at least in part through resistance to apoptosis, exhibit phenotypic and genomic changes characteristic of an EMT, including increased vimentin expression and higher levels of proto-oncogene transcripts as well as elevated pAkt and Bcl-XL [39, 40]. EMT-like transitions have been also been shown to shown to confer resistance to TGF-β induced apoptosis mammary epithelial cells [41, 42]. Evidence from several independent research groups suggests that the EMT-inducing transcription factors Snail and Slug can induce expression of anti-apoptotic genes while down-regulating pro-apoptotic pathways in both epithelial cells and hematopoetic progenitor cells [43–45]. This EMT-related inhibition of apoptosis may provide selective advantage for tumor cells which are transitioning to a mesenchymal-like state. The activation of the PI3K/Akt cell survival pathway through alternate RTKs may protect against TGF-β induced apoptosis while driving pathways critical to carcinogenesis.

RTKs, such as EGFR, c-Met, IGF-1R, FGF receptors and the non-RTK c-Src have been reported to induce phosphorylation of E-cadherin and associated catenins, resulting in their degradation [46], providing a link between oncogenic activation of these kinases and induction of EMT. Thus a rationale exists for the prevention of EMT-like transitions and tumor metastasis through inhibition of these oncogenic kinases in early-stage carcinomas.

IGF-1R signaling and EMT

Several lines of evidence implicate IGF-1R signaling as an important driver of EMT. In mammary epithelial cells, constitutively active IGF-IR caused cells to undergo EMT which was associated with dramatically increased migration and invasion, and this transition was mediated by the induction of Snail and downregulation of E-cadherin [47]. Multiple groups have demonstrated that IGF-1R activation or overexpression correlates with increased invasion and metastasis [48–51]. These effects are mediated, at least in part, by its ligand, IGF-1. IGF-1 is known to influence cell adhesion to the substratum and integrin-mediated cell motility [52]. Furthermore, IGF-1 stimulation can induce the phosphorylation and transcriptional activation of β-catenin and dissociation of E-cadherin from the cell membrane [53]. In addition to disruption of homotypic cell adhesion, IGF-1 has also been shown to promote tumor invasiveness via secretion of matrix metalloproteinases [54] or crosstalk with integrin signaling pathways [55].

IGF-1R is ubiquitously expressed but is frequently overexpressed in tumors, including melanomas, pancreas, prostate and kidney (reviewed in [56]). Perhaps most relevant is not the expression level of IGF-1R but its function in cancer cells. IGF-1R signaling promotes Akt phosphorylation and protection from apoptosis, which is predicted to limit the efficacy of standard of care chemotherapies. Thus there is a strong rationale for development of IGF-1R targeted therapies, and IGF-1R inhibition might be expected to enhance the effect of cytotoxic chemotherapies or other molecular targeted therapies. Several IGF-1R targeted therapeutics are currently in early clinical trials. An array of monoclonal antibodies are currently in Phase I or Phase II trials, including CP-753,871 (Pfizer), AMG0479 (Amgen), R1507 (Genmab/Roche), IMC-A12 (Imclone), AVE-1642 (ImmunoGen/Sanofi-Aventis), MK0646 (Merck) and SCH717454 (Schering-Plough). Two low molecular weight inhibitors of the IGF-1R tyrosine kinase have entered Phase I trials: OSI-906 (OSI Pharmaceuticals) and INSM-18 (Insmed). Given the role of IGF-1R signaling in cell survival and EMT, one might hope that these therapies might target both the primary tumor as well as emerging metastatic cells.

EGFR promotion of EMT-like transitions

EGFR function is frequently dysregulated in epithelial tumors, and EGFR signaling has been shown to play an important role both in cancer progression and in EMT-like transitions. EGF has been shown to promote tumor cell migration and invasion, at least in part through dephosphorylation and inactivation of FAK [57–60]. EGF treatment of tumor cells overexpressing EGFR also leads to downregulation of caveolin-1 which leads to loss of E-cadherin, transcriptional activation of β-catenin and

enhanced invasiveness [61]. Thus inhibition of EGFR might be expected to restrain EMT in certain cellular contexts. In support of this hypothesis, ligand-independent, constitutively active forms of EGFR can increase motility and invasiveness of tumor cells and EGFR inhibitors have been shown to inhibit cancer cell migration in vitro [62–65]. In oral squamous cell carcinoma cells EGFR inhibition resulted in a transition from a fibroblastic morphology to a more epithelial phenotype as well as accumulation of desmosomal cadherins at cell–cell junctions [66]. Taken together, these summarized observations suggest that inhibition of EGFR affects tumor growth through inhibiting EGFR-dependent mitogenic stimulation but may also restrain invasion and metastasis by re-establishing intercellular contacts between tumor cells. Such inhibition of EMT, and potentially metastasis, may translate to improved overall patient survival in the clinical setting.

EGFR inhibitors in cancer therapy

EGFR is widely expressed by cells of both epithelial and mesenchymal lineages, and the degree of EGFR expression is variable [67]. EGFR overexpression has been reported in multiple human cancers including non-small cell lung (NSCLC), head and neck (HNSCC), pancreas, breast and central nervous system (CNS), and has been shown to correlate with poor survival [68]. Several selective EGFR and Her family antagonists have been shown to offer clinical benefit, including erlotinib (OSI Pharmaceuticals/Genetech/Roche), gefitinib (Astra Zeneca) and lapatinib (GlaxoSmithKline). Erlotinib is approved for the treatment of NSCLC patients who have failed two or three previous rounds of chemotherapy. Erlotinib is also approved in the USA and Europe for the treatment of pancreatic cancer in combination with gemcitibine. Lapatinib, a dual inhibitor of EGFR and Her2, has been shown to delay progression of trastuzumab-refractory breast cancer and is used in combination with capecitibine for patients who have received prior therapy with an anthracycline, a taxane, and trastuzumab. Anti-EGFR antibodies have also shown clinical utility, including cetuximab (Imclone/Bristol Myers) and panitumamab (Abgenix/Amgen) which are approved for the treatment of EGFR-expressing, metastatic colorectal carcinoma. Additional small molecule dual EGFR-Her2 inhibitors which bind irreversibly are in earlier stages of clinical development. The current EGFR inhibitors have provided significant clinical benefit when compared to the current standard of care, however not all patients derive a benefit in terms of overall survival or RECIST criteria. For example, in the BR.21 trial which compared the efficacy of erlotinib as a single agent in comparison to placebo in NSCLC patients who did not respond to chemotherapy, the overall response rate was only 8.9% while the hazard ratio for treatment benefit associated with overall survival was 0.7 [69]. The median survival among patients who were treated with erlotinib was 6.7 months compared to 4.7 months for those treated with placebo. These data suggest both that RECIST did not directly correlate with potential survival benefit and that some, but not all, patients clearly benefited from erlotinib. This observation spurred research to identify biomarkers to predict patient response to erlotinib and potentially other EGFR antagonists, and to identify the mechanistic basis for the differential response.

EMT and sensitivity to EGFR inhibitors

Parallel efforts from independent research groups employed gene expression and proteomic profiling to identify biomarkers which correlated with sensitivity to erlotinib [70–72]. Each of these groups used panels of established NSCLC cell lines and xenografts to determine commonalities within those cell lines for which erlotinib inhibited growth in vitro and in vivo, as compared to those cell lines which were insensitive to erlotinib. Those cell lines which were classified as sensitive, having greater than 50% maximal inhibition of proliferation, expressed the canonical epithelial markers E-cadherin and γ-catenin and displayed the classic cobblestone epithelial morphology and tight cell–cell junctions of epithelial cells. Conversely, those cell lines which were relatively insensitive to erlotinib lacked those epithelial markers and expressed proteins characteristic of mesenchymal cells, including vimentin, fibronectin and Zeb-1 and exhibited a more fibroblastic, scattered morphology. These changes are consistent with cells which have undergone EMT. These observations were later extended to other tumor types and EGFR antagonists, including pancreatic, colorectal [5], head and neck [73], bladder [74] and breast [75] suggesting that EMT status may be a broadly applicable indicator of sensitivity to EGFR inhibitors.

An important clinical substantiation of this hypothesis resulted from a retrospective analysis of TRIBUTE, a NSCLC phase III randomized trial which compared the combination of erlotinib with chemotherapy to chemotherapy alone [76]. This trial failed to show significant clinical benefit for the concurrent administration of erlotinib and chemotherapy, however subset analysis of E-cadherin levels in patient samples using immunohistochemistry was revealing. Patients with tumor samples showing strong E-cadherin staining had a significantly longer time to progression (hazard ratio 0.37) and a nonsignificant increase in overall survival when treated with the combination of erlotinib and chemotherapy as

compared to chemotherapy alone [71]. Notably, expression of EGFR itself, as measured by IHC was a poor predictor of response to EGFR antagonists, both in the clinic and in cultured cell lines [70, 77]. Recent studies suggest that it is not the abundance of receptor, but rather the activation of the EGFR signaling axis that mediates sensitivity to EGFR inhibition, at least in vitro [78–80]. Collectively, these compelling in vitro data and clinical findings indicate that expression of E-cadherin or vimentin, and EMT as a process, may be viable biomarkers to predict efficacy of EGFR inhibitors in cancer patients. Clearly further clinical evaluation is warranted and is currently underway at multiple sites.

Bypassing EGFR-dependent activation of PI3-kinase and Ras pathways

The mechanism by which EMT results in insensitivity to EGFR antagonists appears to derive from the acquisition of alternative routes to activation of the PI3 kinase-Akt-mTOR and Ras-Raf-Mek-Erk pathways. It has been reported that cells which have transitioned to a mesenchymal-like state may have upregulated the PI3K-Akt cell survival pathway to circumvent apoptosis [39], and consequently have decreased sensitivity to an inhibitor of the MAPK proliferative pathway. Human cancer cells which exhibit mesenchymal characteristics express lower levels of EGF ligands, suggesting that these cells have become dependent upon alternate signaling pathways [71]. However fetal rat hepatocytes which have undergone TGF-β -mediated EMT have upregulated EGFR ligand expression [81], and in this system inhibition of EGFR does not block TGF-β -mediated EMT [82]. Thus in fetal hepatocytes, EGFR activation appears to be dispensable for the EMT process, however in human cancer cells EGFR signaling is an important driver of EMT. The expression EGFR and reported downregulation of ligand in mesenchymal NSCLC cells suggests that it is not the expression of the growth factor receptor, but rather the usage of that receptor, which governs sensitivity to targeted inhibitors. For example it has been shown that Her3, a heterodimerization partner of EGFR, allows EGFR to effectively activate the PI3 kinase-Akt pathway [83]. It has also been shown that Her3 RNA transcripts and protein are attenuated or lost during EMT-like transitions, depriving EGFR of PI3 kinase coupling [70, 80]. There is substantial evidence that cancer cells can readily shift the cellular equilibrium to rely on alternate growth factor and adhesion signaling pathways in response to EMT-like transitions. For example, inhibition of the Ras-MAPK proliferative pathway can lead to increased activation of the PI3K-Akt cellular survival pathway [75, 84, 85]. Therefore it is not surprising that increased IGF-1R signaling has been associated with insensitivity to EGFR inhibitors [86, 87]. High concentrations of the ligand IGF-1 were shown to inhibit apoptosis caused by the EGFR antagonist erlotinib, possibly due to an increased reliance on the IGF-1R/PI3K signaling axis.

Interestingly, while IGF-1R can promote EMT-like transitions it appears to be less frequently used by mesenchymal-like carcinoma cells as a sole driver of the PI3 kinase pathway. IGF-1R activation drives upregulation of the PI3K-Akt survival pathway and promotes epithelial-to-mesenchymal transition, and these changes may account for the reduced cellular sensitivity to EGFR antagonists. However, committed mesenchymal-like NSCLC, colon and pancreas adenocarcinomas are not dependent on IGF-1R for proliferation or survival [75], suggesting that, like EGFR, IGF-1R signaling is an important driver in epithelial cells and can promote EMT, but once these cells have transitioned to a mesenchymal state they are no longer reliant on IGF-1R. This hypothesis would suggest that the combination of an EGFR antagonist such as erlotinib and an inhibitor of IGF-1R could synergistically inhibit proliferation and potentially drive apoptosis in early stage tumors with an epithelial phenotype. Several in vitro studies have provided data which supports this hypothesis [86, 88–91]. Furthermore, since blockade of either IGF-1R [92, 93] or EGFR [94–96] signaling results in decreased metastasis in vivo, partnering EGFR and IGF-1R antagonists might also improve overall patient survival in early disease in epithelial tumors dependent on EGFR and IGF1R signaling.

Once a cancer cell has transitioned to a mesenchymal-like phenotype, cellular dependence on IGF-1R and EGFR signaling is reduced and alternate growth factor pathways are activated. Recent data suggest that EMT-like transitions can promote the novel acquisition of alternate receptor tyrosine kinase (RTK) autocrine and paracrine loops [97], such as PDGFR, which can exert proliferative and anti-apoptotic actions. PDGFRα and β are restricted to cells of mesodermal origin [98] and autocrine PDGFR signaling has been described in non-epithelial tumors such as gliomas [99, 100]. However high expression has been observed in multiple tumor types including ovarian [101] prostate [102] and breast [103] carcinomas, suggesting that PDGFR may be detectable in stroma and in tumor cells which have undergone EMT. Autocrine PDGFR expression has been shown to promote progression of breast and ovarian cancer and contribute to maintenance of a mesenchymal-like cell phenotype [101, 103, 104]. Thus, mesenchymal cells which have acquired alternate signaling pathways, such as PDGFR, to activate PI3K signaling and prevent apoptosis may be sensitized to specific molecular targeted therapies. The acquisition of these receptor signaling pathways which are predominantly used in

mesenchymal cells may overlap on a common set of required signaling nodes, although this remains to be determined. Nevertheless, this observation provides support for rationally designed combinations of targeted therapies to impede the complex, heterogeneous signaling pathways that exist within a tumor.

In summary, we propose a model in which carcinomas undergo an epithelial-to-mesenchymal-like transition, potentially triggered by dysregulated growth factor signaling or inflammation-mediated activation of the PI3 kinase and Ras pathways [105]. Tumor cells with a mesenchymal-like phenotype become less reliant on EGFR, IGF-1R, Met and Ron signaling pathways. Over time, mesenchymal-like tumor cells appear to become more committed to a mesenchymal phenotype through epigenetic changes and can upregulate alternate receptor tyrosine kinase pathways as mechanisms for survival signaling and escape from anoikis. The interactions of EGF receptor signaling with other cellular pathways regulating mitogenic, survival and migration cues has clinical implications as we try to identify and develop treatments which not only target the primary tumor cells but also the mesenchymal-like cells deriving from EMT-like transitions that can promote cancer metastasis and recurrence.

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