The role of epithelial to mesenchymal transition in resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small cell lung cancer

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Abstract: Inhibition of the epidermal growth factor receptor (EGFR) is an important strategy when treating non-small cell lung cancer (NSCLC) patients. However, intrinsic resistance or development of resistance during the course of treatment constitutes a major challenge. The knowledge on EGFR-directed tyrosine kinase inhibitors (TKIs) and their biological effect keeps increasing. Within the group of patients with *EGFR* mutations some benefit to a much higher degree than others, and for patients lacking *EGFR* mutations a subset experience an effect. Up to 70% of patients with *EGFR* mutations and 10–20% of patients without *EGFR* mutations initially respond to the EGFR-TKI erlotinib, but there is a severe absence of good prognostic markers. Despite initial effect, all patients acquire resistance to EGFR-TKIs. Multiple mechanisms have implications in resistance development, but much is still to be explored. Epithelial to mesenchymal transition (EMT) is a transcriptionally regulated phenotypic shift rendering cells more invasive and migratory. Within the EMT process lays a need for external or internal stimuli to give rise to changes in central signaling pathways. Expression of mesenchymal markers correlates to a bad prognosis and an inferior response to EGFR-TKIs in NSCLC due to the contribution to a resistant phenotype. A deeper understanding of the role of EMT in NSCLC and especially in EGFR-TKI resistance-development constitute one opportunity to improve the benefit of TKI treatment for the individual patient. Many scientific studies have linked the EMT process to EGFR-TKI resistance in NSCLC and our aim is to review the role of EMT in both intrinsic and acquired resistance to EGFR-TKIs.

Keywords: Epidermal growth factor receptor (EGFR); epithelial to mesenchymal transition (EMT); non-small cell lung cancer (NSCLC); tyrosine kinase inhibitor (TKI)

Epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) and resistance to lung cancer therapy

Non-small cell lung cancer (NSCLC) is a leading cause of cancer-related death worldwide. The 5-year survival remains at 10–15% despite advances in treatment options.

Erlotinib, a TKI targeting EGFR, was initially explored as a second-line treatment in NSCLC patients progressing on standard chemotherapy. Survival was prolonged compared to placebo (1). However, erlotinib was soon discovered to be especially efficient in patients with a mutation in the tyrosine kinase domain of *EGFR*. These patients are now
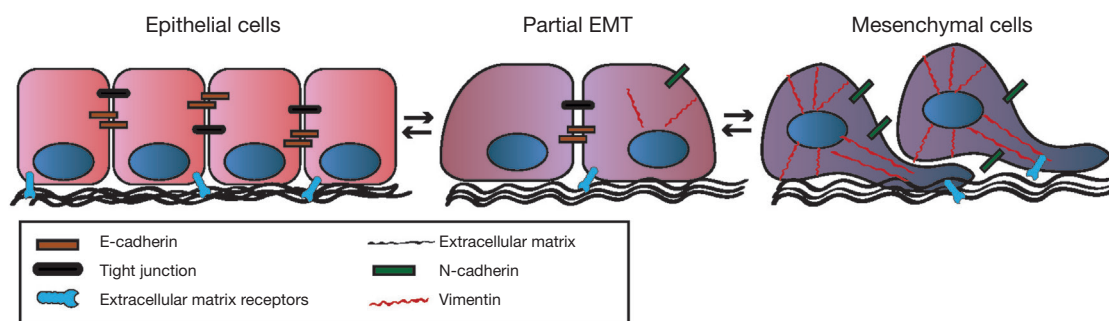
Figure 1 EMT induces a cellular phenotypic shift. Epithelial cells are characterized by the presence of apico-basal polarity, intact cell-cell contacts, barrier function, and lack of movement. During the EMT process, cells with partial EMT appear characterized by the incipient presence of a front-rear polarity and have leaky cell-cell contacts. At the same time upregulation of the mesenchymal markers vimentin, N-cadherin, ZEB1 and 2, SNAIL, SLUG and matrix metalloproteinases begin. In addition, these partial EMT cells express lower amounts of E-cadherin and ZO-1 than epithelial cells. After finalization of EMT, the established mesenchymal cells have a clear front-rear polarity and no or few cell contacts. The expression of mesenchymal proteins is increased, and a cadherin gene-expression shift, most often from E-cadherin into N-cadherin expression, appears. EMT, epithelial to mesenchymal transition.

offered EGFR-TKIs such as erlotinib and gefitinib as first-line treatment (2-4). EGFR-TKIs show an impressive response rate in *EGFR*-mutated patients with 70% having an objective response to treatment (5-8). Unfortunately, all patients acquire resistance over time. So far, some resistance mechanisms have been discovered including the T790M secondary mutation in *EGFR*, *MET* amplification, *FGFR* overexpression, *IGF1R* overexpression, transition to small cell lung carcinoma, gain of cancer stem-cell features, and epithelial to mesenchymal transition (EMT), but much still needs to be revealed (9-13). Up to 30% of the patients with *EGFR* mutations experience no objective response to EGFR-TKIs and hence appear as intrinsic resistant (14,15). Some patients without *EGFR* mutations initially benefit from erlotinib with experience of objective response (approx. 3%) or stable disease (approx. 25%) (3,16). Among *EGFR* wild-type patients, *KRAS* mutations have been proposed as a *de novo* resistance mechanism to EGFR-TKIs although the results are not unambiguous (17,18).

An urgent question is the elucidation of the molecular mechanisms behind development of TKI resistance in NSCLC since this could hold important implications for development of improved therapeutic protocols. In recent years, results assigning EMT as a candidate mechanism for mediating especially acquired EGFR-TKI resistance have emerged. Since EMT is a reversible transcriptionally regulated phenotypic shift, understanding the dynamics underlying EMT represents promising novel perspectives in therapeutic treatment of NSCLC.

Epithelial to mesenchymal transition (EMT)

EMT is a process involved in normal embryogenesis (19). Epithelial cells appear with a distinct apical versus basolateral polarity established by dense, strong adherence and tight junctions. Epithelial cells serve as surface barriers and exercise secretory functions. During embryonic development some differentiated, polarized, epithelial cells undergo morphological changes as a response to extracellular stimulation. This process is referred to as EMT (*Figure 1*). The EMT process is reversible and mesenchymal-to-epithelial transformation is also a well-characterized process (19). Furthermore, EMT hold implications in cancer processes such as invasion and metastasis seen in most types of solid tumors (20). The acquirement of this invasive potential of tumor cells through EMT is interrelated with the formation of migratory, mesenchymal cells with loose cell-cell contacts and apico-basolateral polarity (21). EMT also leads to upregulation of anti-apoptotic signals rendering the cells more tumorigenic and less responsive to treatment (22,23).

EMT inducers, such as transforming growth factor-β (TGFB1) or receptor tyrosine kinase (RTK) ligands trigger changes in gene expression by activating PI3K, mitogen activated protein (MAP) kinase, RHOA and SRC signaling networks. As a consequence, transcriptional repressors like the zinc-finger proteins SNAIL (SNAI1), SLUG (SNAI2), ZEB1 and ZEB2, as well as the basic helix-loop-helix factors TWIST and E47, are upregulated (24). SNAIL, SLUG, ZEB1 and ZEB2 direct EMT by binding to E-box

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elements in transcriptional regulatory regions of response genes, e.g., the promoter of the gene *CHD1* encoding the adherence junction-protein E-cadherin. By recruiting histone deacetylases (HDACs) and other co-repressors, the EMT mediators facilitate chromatin condensation and transcriptional repression of *CHD1*. E-cadherin may also be depleted through cleavage by metallo-proteases (25,26). When E-cadherin expression is reduced, adherence junctions are broken and the cells abrogate their cell polarity. This is accompanied by an increase in vimentin and N-cadherin expression (*Figure 1*).

E-cadherin depletion also leads to accumulation of the adherence junction component beta-catenin in the cytoplasm (27). In cooperation with Wnt-mediated signaling, beta-catenin forms a transcriptional complex with TCF/LEF and localizes to the nucleus (28). Beta-catenin/LEF here promotes gene-expression changes initiating or supporting EMT.

Complex gene-expression changes are characteristic for the EMT program and epithelial and mesenchymal cells can be distinguished by expression of a number of classical markers used for defining either epithelial or mesenchymal cells (29). Classical epithelial markers include adherence and tight-junction proteins such as E-cadherin and ZO-1, whereas the mesenchymal counterparts are frequently characterized by N-cadherin, fibronectin and vimentin (30).

EMT and treatment response to EGFR-TKIs

The EMT status has been explored as a marker in both prognosis of NSCLC and as a predictor of therapy response. It has been debated whether expression of EMT markers can predict the overall prognosis of the disease. One study performed on 95 surgically resected lung adenocarcinomas of all stages (53.7% stage I–II and 46.3% stage III–IV) found the degree of alteration of epithelial and mesenchymal markers to be significantly associated with a poor outcome (31). Another study, however, found no correlation between EMT and the disease free survival of 183 surgically resected stage I–III lung adenocarcinomas (32). The later study primarily included early stage patients (89.6% stage I–II and 10.4% stage III), which may explain the differences observed in the two studies. Another study found EMT marker status in the primary tumor to be correlated with metastatic burden (33), and vimentin expression has been shown to correlate with higher TNM status (31). Hence, EMT markers may primarily have prognostic value in later more advanced stages of NSCLC.

Since EMT is a dynamic process and tumors are prone to change from epithelial to mesenchymal in adaptation to therapy, the initial EMT status may be altered as a consequence of several rounds of treatment. The alkylating chemotherapeutic agent cisplatin, for instance, can induce EMT and confer resistance to subsequent gefitinib treatment (34). In reflection of the EMT interchangeability, Shintani *et al.* reported that a change towards a mesenchymal status during chemotherapy and radiation treatment was correlated with a lower disease free survival rate (35). Hence, the process and evolution of EMT in initial lines of treatment may be important for predicting the response to subsequent treatment such as EGFR-TKIs. Therefore, the inclusion of EMT status analyses during treatment could be an important clinical supplement for monitoring treatment response. Furthermore, EMT and EGFR-TKI sensitivity may be directly connected. Witta *et al.* reported how E-cadherin expression potentiated the sensitivity to and the apoptotic effect of gefitinib. Thus, EMT induced E-cadherin repression can directly influence and change the EGFR-TKI sensitivity (36).

Intrinsic resistance in EGFR mutated patients

The presence of *EGFR* mutations and an epithelial phenotype is correlated (37,38). Ren *et al.* found that among 99 mutated patients 57.7% appeared with epithelial or intermediary epithelial-to-mesenchymal phenotype, whereas only 25.77% was clearly mesenchymal (38). The predictive value of EMT status in *EGFR* mutated patients was also investigated, but no significant correlation between an epithelial phenotype and EGFR-TKI response was identified in the group of *EGFR* mutated patients (37,38). *EGFR* mutated patients in general experience a good relative response to EGFR-TKI with only 30% of patients not responding (14,15). This taken together with the tendency to an epithelial phenotype among the *EGFR* mutated patients suggests that the presence of an *EGFR* mutation overrules any influence of EMT status.

Other factors not related to mesenchymal status may explain the lack of response in some *EGFR* mutated patients. First, low expression of the pro-apoptotic BCL-2 family member BIM associates with an inferior response to EGFR-TKIs, but has no influence on chemotherapy response (39). Second, an explanation may also be low-level frequency of T790M mutations prior to treatment giving rise to intrinsic resistant clones (39). This is however still debated. Third, genetic polymorphisms including SNPs and

differential lengths of CA-repeats within the *EGFR* gene can influence the response to EGFR-TKIs preferentially in *EGFR* mutated patients (40,41).

Treatment response in EGFR wild-type patients

Tumors from *EGFR* wild-type NSCLC patients constitute a biologically different group than the *EGFR* mutated tumors. Since *EGFR* mutated tumors often have an epithelial phenotype (37,38), *EGFR*-driven cancer may have an inherent tendency towards being epithelial. The subgroup of *EGFR* wild-type patients responding to EGFR-TKIs exploits another type of EGFR-dependency that may also be linked to an epithelial phenotype. In consequence, another EGFR-independent subgroup exists, which may be driven by a mechanism related to a mesenchymal phenotype. Hence, EMT status may be a valuable marker for predicting EGFR-TKI treatment response in the *EGFR* wild-type group. This hypothesis has been investigated and confirmed in several *in vitro* and *in vivo* studies (36-38,42-44). One example is a retrospective analysis of 99 patients with wild-type *EGFR* where EMT status was evaluated using a combined IHC-score for E-cadherin, N-cadherin, fibronectin and vimentin and showing that EMT status is a predictive factor for experiencing an objective response or stable disease during EGFR-TKI treatment (epithelial 23.5%, mesenchymal 2.4%) (38).

Acquired resistance in EGFR mutated patients

All initially EGFR-TKI sensitive patients will develop resistance, and alterations in cell signaling during this process may include a switch from an epithelial to a mesenchymal phenotype as one participating mechanism. However, the general lack of tumor biopsy material, representing the temporal resistance progression, limits studies of acquired resistance using clinical samples. But, some highly valuable studies have been made. Sequist *et al.* studied re-biopsies taken from 37 *EGFR*-mutated patients treated with erlotinib or gefitinib (13). For this study biopsies were taken at the time of progression according to the RECIST criteria. Among the 37 re-biopsies they observed transition to SCLC (5), MET amplification (3), EGFR T790M mutations (21), *EGFR* amplification (4) and EMT (3). Some cases showed heterogeneity with co-occurrence of T790M and other alterations such as *EGFR* amplification. In seven cases, no genetic alteration was found, and in 3 of these cases EMT was detected. Evaluation of EMT was only performed in

the seven re-biopsies without known resistance mechanism and in 5 of the re-biopsies presenting with EGFR T790M mutations (13). Similarly, EMT was not investigated in a large re-biopsy study performed on 155 patients (45).

Since only a few re-biopsies have been evaluated for EMT, it is difficult to estimate a quantitative measure of the general occurrence of EMT alone or accompanying other resistance mechanisms. Thus, the occurrence of EMT could in principle be much higher than reported so far. Notably, EMT were reported to co-occur with EGFR T790M mutations in another study using re-biopsies (46), and was also evident in a metastatic lesion at time of progression in a Korean case study (47). As a contrast to the sparse investigation of EMT as an acquired resistance mechanism in EGFR-TKI treated patients, numerous cell studies exploring and highlighting the importance of EMT in TKI resistance development exists as elaborated below.

Molecular mechanisms for EMT induction related to EGFR-TKI resistance

EMT can be induced and regulated by various growth and differentiation pathways and factors. These include Wnt and Notch proteins, NFκB pathway, TGF-β, and RTKs and activating growth factors: IGF1, GAS6, HGF, FGF and EGF. Several links between EMT-induction and EGFR-TKI treatment exists (*Figure 2*). In the instance of Notch-induced EMT, Notch is activated during EGFR-TKI treatment as EGFR signaling normally has a repressive effect on this pathway (48).

Moreover, EGFR-TKI treatment can create a selective pressure favoring cells capable of bypass signaling through activating of RTKs or TGFbR and subsequent activation of SMAD, PI3K/AKT and MAP kinase pathways. Co-occurring induction of EMT may result in generation of mesenchymal-like cells which have a growth and survival advantage do to the lack of E-cadherin expression and through the anti-apoptotic effect of the EMT factors SNAIL, SLUG and ZEB1 (22,23,49).

TGF-β

Cancer cells can increase their production of active TGF-β during development of EGFR-TKI resistance. TGF-β triggers EMT and allow the cells to become invasive (50) (*Figure 2*). Furthermore, transient TGF-β-stimulation induces EMT and acquired resistance to EGFR-TKIs in NSCLC cell lines (51-53). TGF-β is the most studied
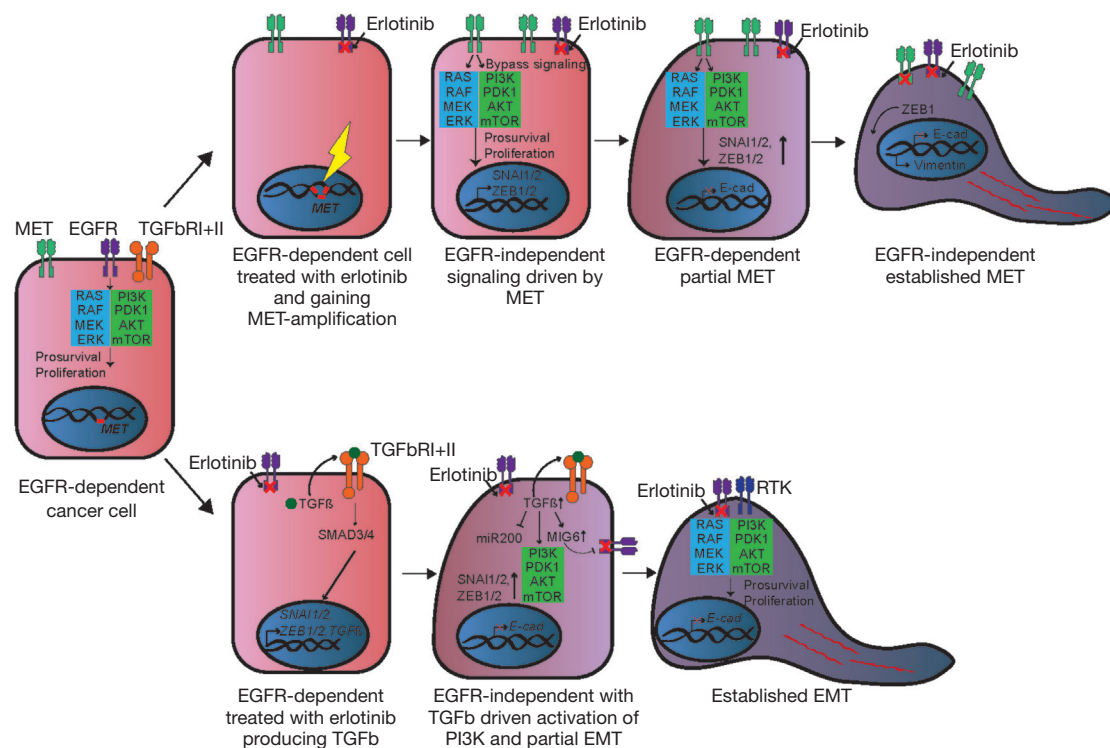
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Figure 2 Models for induction of EMT during erlotinib resistance development. Above: erlotinib treatment induces increased IGF1R activation, which restores signaling through PI3K/AKT and MAPK pathways. Restoration of the signaling triggers transcription of mesenchymal transcription factors and leads to an IGF1R-dependent partial or full EMT. Eventually some cells might only depend on EMT signals in order to survive and hence no longer rely on IGF1R-signaling. Below: most cancer cells produce TGF-β, but TGF-β has both tumor-promoting and tumor-suppressing roles at early stages. When the cells are treated with erlotinib, the balance may shift so that cells primarily benefit from the tumor-promoting TGF-β signals. TGF-β initiates EMT through inhibition of miR-200 family and hence upregulation of mesenchymal transcription factors. In addition, TGF-β upregulates the EGFR repressor MIG6 and restores PI3K/AKT signaling. Secondly, PI3K/AKT signaling can activate a kinase shift, giving rise to the expression of another RTK. Hereby, MAPK signaling is restored and EMT solidified (40). EMT, epithelial to mesenchymal transition; TGF-β, transforming growth factor-β; EGFR, epidermal growth factor receptor; RTK, receptor tyrosine kinase.

inducer of EMT and initiates the process primarily through activation of the SMAD-pathway. Upon ligand-binding to the TGF-β receptor complex, SMAD2 and SMAD3 are activated through phosphorylation by TGFbRI (54). SMAD4, consecutively, forms a trimer with SMAD2 and SMAD3 and translocates into the nucleus. Through interaction with other DNA-binding transcription factors, SMAD4 represses transcription of epithelial genes such as *CDH1* or activates transcription of mesenchymal genes (55). Several downstream transcription factors including SNAIL, SLUG, ZEB1, ZEB2 and basic helix-loop-helix family members are activated and mediate further downstream effects (50). In addition, TGF-β may also activate the PI3K/AKT and MAP kinase pathways. Although TGFbR is a

serine-threonine kinase, a few tyrosine residues are present. When these are phosphorylated, they create a docking site for Src homology-2-domain containing proteins, like PI3K and GRB2, creating a link to the PI3K/AKT and MAP kinase pathways (56). PI3K/AKT and MAPK activation by TGF-β can further be potentiated by simultaneous stimulation with additional growth factors (54,57).

Receptor tyrosine kinases (RTKs)

RTKs predominantly signal through the PI3K/AKT and MAP kinase pathways. Activation of the PI3K/AKT cell survival pathway protects against apoptosis while driving pathways critical to carcinogenesis (58). MAP kinase,

or, signaling primarily stimulates mitosis and hence cell proliferation (59). In addition, RTKs, such as MET, IGF-1R, FGFRs and the non-RTK c-Src have been reported to induce phosphorylation of E-cadherin and associated catenins, resulting in their degradation (60). Bypass-signaling through activation of a distinct RTK is often seen during acquired EGFR-TKI resistance (61). Although EMT can be induced by RTKs, the process may later be independent of RTK signaling (62). Different stages of EMT exist, including a partial EMT stage with cell contacts, but upregulation of mesenchymal markers. During treatment with EGFR-TKIs EMT may be initiated in the tumor cells, resembling a partial EMT. This could be caused by increased expression of TGF-β (52). Surprisingly, drug-withdrawal may further enhance the EMT state due to an EGFR-mediated boost of a partial EMT existing in a "ready-to-go" fashion (63). A recent study suggested a role for TGF-β in mediating the RTK switch with accompanying activation of AKT and EMT, hence, rendering cells independent of EGFR signaling (52). An increase in TGF-β during resistance development could create an autocrine loop, linking the kinase switch and EMT. Due to the lack of studies on biopsies from patients developing EGFR-TKI resistance, there is limited data on the co-occurrence of kinase switch and EMT *in vivo*. Moreover, due to limited biopsy material, EMT has in most cases only been studied when no other resistance mechanism appeared (13). Below is the relation between EMT in lung cancer and the RTKs IGF1R, AXL, and MET specifically highlighted.

IGF1R

From *in vitro* experiments, it is known that EMT can be initiated by stimulation with IGF1 and reversed when adding the IGF1R inhibitor AEW541 (64). IGF1R activation is also a proposed resistance mechanism to EGFR TKIs in NSCLC cell lines (53,65). In PC9 NSCLC cells made erlotinib-resistant *in vitro*, EMT was reversed upon IGF1R inhibition with I-OMe-AG538 indicating a role for IGF1R in TKI resistance related EMT (53). A study on IGF1R overexpressing cells highlighted the EMT transcription factor SNAIL as the downstream effector of IGF1R-induced EMT (66).

Our own studies (unpublished results) and studies from others suggest a role for IGF1R in EMT initiation, but also that established mesenchymal-like lung adenocarcinoma cells no longer depended on IGF1R signaling for proliferation or survival (67). This indicates that IGF1R signaling can induce EMT, but once these cells have

transitioned to a mesenchymal state they are not necessarily reliant on IGF1R signaling (*Figure 2*).

AXL

AXL belongs to the TYRO3, AXL and MERTK (TAM) family of RTKs characterized by having two immunoglobulin-like domains as well as fibronectin repeat domains in the extracellular region. AXL and the ligand GAS6 are reported as overexpressed in cancer (68). Upregulation of the RTK AXL has also been implicated in acquired erlotinib resistance *in vivo* and *in vitro*, and AXL inhibition comprises a promising target for restoring erlotinib sensitivity (42,69). In a HCC827-derived model, erlotinib-resistant cell clones upregulated AXL with subsequent EMT (69). AXL activation restored AKT, MAPK and Notch signaling while inhibition with RNAi or molecular inhibitors restored erlotinib sensitivity and decreased signaling (69). The knockdown of vimentin likewise restored erlotinib sensitivity and decreased AXL expression, and since no changes were seen in vimentin levels upon AXL inhibition, the state of EMT have become independent of AXL signaling (69). Alternatively AXL upregulation may be an event downstream of EMT.

MET

MET, also called c-met and hepatocyte growth factor receptor (HGFR), has HGF as the only known ligand. MET is normally expressed by cells of epithelial origin, while expression of HGF is restricted to cells of mesenchymal origin (70). HGF mediated EMT induces resistance to chemotherapy in SCLC (71), but so far no reports on MET induced EMT and resistance to EGFR-TKIs in NSCLC exist. Upon HGF stimulation, MET induces several biological responses related to invasive cell growth and abnormal MET activation in cancer correlates with poor prognosis. HGF and MET dependent activation of the MAPK pathway leads to c-FOS mediated upregulation of *EGR-1* transcription. EGR-1 is a positive transcription factor for SNAIL, implicating *SNAIL* upregulation as a core event in HGF-induced EMT (72). *MET* gene amplification is one of the most frequently reported resistance mechanisms in NSCLC, and it will be important to identify if EMT contributes to the MET-amplification-mediated resistant phenotype or if resistance mechanisms are mutually exclusive.

Drug mediated repression of EMT in NSCLC

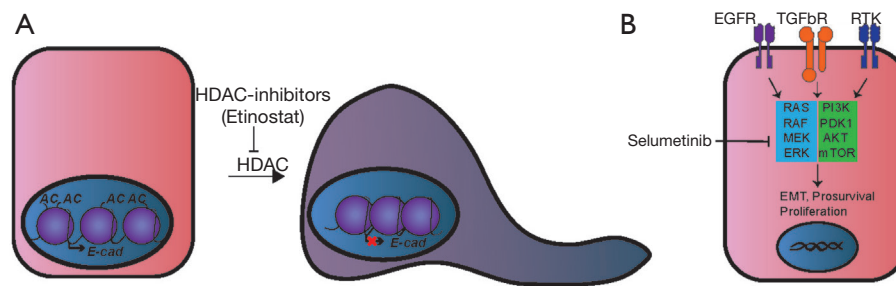In addition to the studies addressing EMT as a potential

Figure 3 Inhibition of EMT in NSCLC cells. (A) The HDAC inhibitor entinostat maintains an open chromatin structure and the transcription of E-cadherin. Entinostat hereby enforces the epithelial state and prevent EMT; (B) selumetinib inhibits MEK and thus the MAPK pathways. As the MAPK pathway is involved in receptor tyrosine kinase signaling as well as TGF-β signaling, selumetinib has potential both as a first line treatment and as a treatment upon resistance development. EMT, epithelial to mesenchymal transition; NSCLC, non-small cell lung cancer; HDAC, histone deacetylase; TGF-β, transforming growth factor-β.

resistance mechanism in NSCLC, several studies have also addressed the potential therapeutic targeting of EMT. Two such EMT targeting drug-types under investigation are HDAC inhibitors [e.g., entinostat (MS-275)] and MEK-inhibitors (e.g., selumetinib) (*Figure 3*). Entinostat abolishes the deacetylation of histones and accordingly helps keeping chromatin in an active, transcribed form. As E-cadherin transcription is regulated through epigenetics, HDAC-inhibitors may induce increased E-cadherin transcription and mesenchymal to epithelial transition (73). In addition, entinostat increases the effect of erlotinib in resistant cell lines (36). This finding led to a phase II clinical trial for entinostat and erlotinib co-treatment. No difference in progression-free survival (PFS), overall survival, or objective response was observed by combining entinostat with erlotinib for the entire group of patients (74). There was, however, a prolonged PFS for patients expressing high levels of E-cadherin at the beginning of the trial. This could indicate that entinostat delays the onset of acquired resistance to erlotinib by preventing the establishment of resistant EMT subclones by keeping the cells in an epithelial state and calls for further biomarker-driven studies addressing this (74).

Pre-treatment of cells with the MEK-inhibitor selumitinib reverses EMT and sensitize cells to EGFR inhibitors (75). Interestingly, selumetinib was effective in inhibiting TKI-resistant Calu-3 derived NSCLC cells as well as the parental Calu-3 cells, emphasizing the central role of MEK in intracellular signaling (67). A recent clinical phase 2 trial of selumetinib together with the anti-mitotic chemotherapeutic docetaxel showed improved PFS for KRAS mutated stage IIIb–VI lung cancer patients (76). Inhibition of MEK with targeted drugs like selumetinib hereby shows promising

potential in patients with inferior EGFR-TKI response rates such as patients with KRAS mutations or with acquired resistance including EMT related cases.

Conclusions

EMT appears an important event in development of TKI resistance in NSCLC cells, and numerous studies show how EMT influences the EGFR-TKI treatment response. Both intrinsic and acquired resistance to EGFR-TKIs can be influenced by the EMT-status of the tumor and this accordingly represents an important factor both for prognosis and selection of treatment protocol.

Acquired resistance to EGFR-TKIs has proven to be a highly circuitous event. Multiple mechanisms exist, and heterogeneity resulting in co-occurrence of different resistance mechanisms is far from unusual. EMT is one such mechanism to be explored both as an independent mechanism as well in the context of other resistance mechanisms. Due to the reversibility of EMT, pharmaceutical targeting of EMT provides hope for counteracting both intrinsic and acquired NSCLC resistance resulting in a higher response rate for EGFR-TKIs and accordingly superior patient survival.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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