Coregulation of pathways in lung cancer patients with EGFR mutation: therapeutic opportunities

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Epidermal growth factor receptor (EGFR) mutations in lung adenocarcinoma are a frequent class of driver mutations. Single EGFR tyrosine kinase inhibitor (TKI) provides substantial clinical benefit, but almost nil radiographic complete responses. Patients invariably progress, although survival can reach several years with post-treatment therapies, including EGFR TKIs, chemotherapy or other procedures. Endeavours have been clinically oriented to manage the acquisition of EGFR TKI-resistant mutations; however, basic principles on cancer evolution have not been considered in clinical trials. For years, evidence has displayed rapidly adaptive mechanisms of resistance to selective monotherapy, posing several dilemmas for the practitioner. Strict adherence to non-small cell lung cancer (NSCLC) guidelines is not always practical for addressing the clinical progression that EGFR-mutant lung adenocarcinoma patients suffer. The purpose of this review is to highlight regulatory mechanisms and signalling pathways that cause therapy-induced resistance to EGFR TKIs. It suggests combinatorial therapies that target EGFR, as well as potential mechanisms underlying EGFR-mutant NSCLC, alerting the reader to clinical opportunities that may lead to a deeper and more durable response. Molecular reprogramming contributes to EGFR TKI resistance, and the compiled information is relevant in understanding the development of new combined targeted strategies in EGFR-mutant NSCLC.

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BACKGROUND

Epidermal growth factor receptor (EGFR) mutations, such as in-frame deletion of the amino acids LREA within exon 19 and L858R substitution mutation, activate EGFR, leading to proliferation and anti-apoptotic signalling. Lung adenocarcinoma (LUAD) patients harbouring such EGFR gain-of-function mutations are sensitive to EGFR tyrosine kinase inhibitors (TKIs). LUAD patients benefit from single therapy with EGFR TKI, leading to tumour shrinkage and prolonged progression-free survival (PFS) and overall survival (OS). Notwithstanding, all patients with TKI-sensitising EGFR mutations will recur after the initial response to TKIs. Adaptive resistance to standalone receptor tyrosine kinase (RTK) inhibitors invariably occurs via kinase signalling rewiring in multiple forms of cancer [1]. Loss of the protein tyrosine phosphatase PTPN12 activates diverse RTKs, such as EGFR and HER2, in triple-negative breast cancer [2]. PTPN12 in triple-negative breast cancer acts as a negative regulator of HER2/EGFR and other TKs, such as PDGFR-β and ABL (Abelson murine leukaemia viral oncogene). Lapatinib in combination with sunitinib reduces the proliferation in triple-negative breast cancer cells [2]. The F-box protein β-transducin repeat-containing protein (β-TRCP) negatively regulates the REST tumour suppressor that positively regulates PTPN12 levels, partly by inhibiting miR-124. PTPN12 is deleted (on chromosome 7) in lung cancer (13.8%) and miR-124 is focally amplified (minimal region of amplification in chromosome 20) in breast and lung cancer (20.1% and 12.1%, respectively). Several lung cancer cell lines (i.e. PC9 exon 19 deletion) harbour focal amplification in chromosome 20 (miR-124). Interestingly, the EGFR Y1148 residue showed the strongest differential phosphorylation (>2-fold) in response to PTPN12 depletion [2]. The use of RTK inhibitor combinations to simultaneously target several RTKs has been suggested as a strategy for personalised cancer therapy. Single RTK inhibitors did not induce the activation of new RTKs, but only increased the phosphorylation of the existing activated RTKs to compensate for the reduced phosphorylation of ERK or Akt [3]. A proposed model for alternative activation of RTKs upon activation of mesenchymal-epithelial transition (MET), by its ligand HGF (from stromal cells), induced interreceptor crosstalk with integrin beta-4 (ITGB4), erythropoietin-producing hepatocellular (EphA2), CUB

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domain-containing protein-1 (CDCP), AXL and Jak1, providing an alternative signalling mechanism and, henceforth, circumventing EGFR TKI effect [4]. We previously found that gefitinib or osimertinib activates STAT3 and Src-YAP1 (Yes-associated protein 1) in EGFR mutation-positive non-small cell lung cancer (NSCLC) cells [5] and that genetic or pharmacologic inhibition of Src or SFKs diminishes YAP1, AXL and CDCP1 phosphorylation or expression. In two cohorts of EGFR mutation-positive NSCLC patients treated with EGFR TKI, we identified that a risk model combining AXL and CDCP1 messenger RNA (mRNA) expression was strongly associated with PFS (hazard ratios of 2.95 and 2.19) and OS (hazard ratios of 3.56 and 2.96) between high- and low-risk group. We postulated that Src-YAP1 signalling leads to further activation of AXL, CDCP1 and MET [6]. Activated AXL has been associated with EGFR and HER3 in maintaining cell survival and inducing drug-tolerant cells to osimertinib [7]. Via other RTKs, parallel bypass pathways have been comprehensively reviewed by Rotow and Bivona [8] and by Tomasello et al. [9]. RTK phosphorylation/activation profiles have been analysed from different tissue origins, including lung cancer. Similar results were obtained from the cancer cell lines, the primary cancer samples and the xenograft samples that had more than one RTK activated [3]. The mechanism of activation of multiple RTKs is multifarious, including phosphatase PTPN12 that serves as a feedback mechanism to limit receptor signalling in triple-negative breast cancer. The suite of RTK substrates inhibited by PTPN12 includes EGFR and others [2, 10]. New data indicate that targeting AXL expressed on tumour cells and MERTK in the tumour microenvironment is predicted to enhance immuno-therapy activity. Tumour-associated M2 macrophages express MET and the inhibition of MERTK blocks the immune-suppressive effects of macrophage efferocytosis [11]. Adding further evidence, GAP6/MERTK signalling has been seen to stimulate NSCLC proliferation. Nuclear PD-L1 regulates the synthesis of GAS6 mRNA, promoting GAS6 secretion to activate MERTK signalling pathway [12]. Interestingly, epiregulin (an EGFR ligand) is predominantly expressed in macrophages in the tumour microenvironment. Epiregulin secreted by macrophages induces the formation of the EGFR/ERBB2 heterodimer and causes resistance to EGFR TKI in an EGFR/ERBB2-AKY axis-dependent manner [13].

Assessment of clinical specimens has shown a ceaseless number of resistance mechanisms to either first- or second-generation EGFR TKIs (i.e. erlotinib, afatinib) or third-generation EGFR TKIs (i.e. osimertinib) that have principally included up-regulation of AXL [7, 14], Her-2, MET, Akt [15], ERK and nuclear factor-kB (NF-kB) signalling [16]. Similarly, EGFR second-site mutation T790M often reappears post osimertinib treatment, while other EGFR mutations are acquired, such as EGFR C797S, G724, L792 and L718/G719 [17, 18]. Variagation of other genetic defects has been found following first-line osimertinib or later-line osimertinib, such as RET fusions, BRAF fusions, K-Ras mutations, MET amplification and MET H109Y mutations, EGFR amplification and histological transformation to small cell lung cancer and squamous cell carcinoma [17]. Therefore, the physician is confronted with a broad array of resistance mechanisms either pretreatment or post-progression in the analysis of tumour samples and circulating tumour DNA in the plasma. Next-generation sequencing (NGS) platforms, as well as custom RNA-sequencing panels, provide extensive information on each individual EGFR-mutant lung cancer patient. Minion combinatorial therapies are being developed and signalling targetable pathways have been charted [19].

**PRKCD** (protein kinase Cδ) a mediator of TKI resistance

EGFR TKIs, such as gefitinib, induced EGFR heterodimers in TKI-resistant EGFR-mutant NSCLC, where phosphorylation of EGFR Y845 and Y1086 was almost suppressed. However, EGFR pY1173 was only partially reduced by gefitinib and promoted activation of phospholipase v2 (PLCv2) and PKCδ when sustained by EGFR heterodimer [20] (Fig. 1a). Phosphatase and tensin homologue (PTEN) loss [21] and Akt and NF-κB pathway activation are present in H1650 cells (EGFR-del19 mutation) resistant to TKIs [16]. Intriguingly, EGFR depletion attenuated all Akt, ERK and ReI Akt phosphorylation compared with EGFR kinase inhibition, which did not affect Akt or ReI. Similar effects of EGFR depletion were noted in two other NSCLC cell lines, H1975 (EGFR L858R/T790M mutation) and H820 (EGFR-del19/T790M mutation and MET amplification) [20]. Of the 15 HCCB27 gefitinib-resistant clones (median inhibitory concentration IC50 > 1 μM), none had the T790M mutation, but showed attributes of resistance, such as up-regulation of Her-2, AXL, ERK, Akt or NF-κB in western blot analysis [20], indicating heterogeneous mechanisms of TKI resistance. PKCδ was identified as a common mediator involved in the TKI-insensitive EGFR pathways. TKI-resistant H1650 cells treated with a PKC inhibitor (sotrastaurin) or phospholipase C (PLC) inhibitor (U73122) displayed strong synergism (CI < 0.3) with gefitinib. The results demonstrated that PKCδ and PLCβ2 are implicated in the TKI-insensitive EGFR pathways [20]. PKCδ nuclear localisation was readily detectable in a TKI-resistant EGFR-mutant H1650 model. Immunofluorescence staining showed that EGFR depletion, but not kinase inhibition, reduced nuclear PKCδ. As a membrane-bound receptor, EGFR interacts with other RTKs, Her-2 and AXL, which have been previously implicated in PKCδ activation [22]. Further analysis showed that interaction between EGFR and AXL or Her-2 may sustain EGFR Y1173 phosphorylation in resistant cells treated with TKI. When phosphorylated, EGFR Y1173 functions as a docking site for PLCγ (Fig. 1a). Such findings illustrate that EGFR-Y1173-PLCβ2-nuclear PKCδ is a common axis of resistance mediated by TKI-insensitive EGFR pathways [20]. Schematic representation of the autophosphorylation sites in the EGFR and activation of the corresponding major signal transduction pathways were reported by Sordella et al. [23]. PKCδ is phosphorylated on tyrosine 374 (Y374) as a substrate of the non-receptor tyrosine phosphatase and tumour suppressor PTPN14 (Pez). Also, RIN1 (Ras and Rab interactor 1) is a binding partner of PTPN14. Loss of catalytically functional PTPN14 increases the abundance of EGFR at the cell surface of breast cancer cells. Patient survival was worse when breast cancer tissue had increased expression of the genes encoding RIN1 or PRKCD [24]. The non-RTK, feline sarcoma-related (FER), phosphorylates PKCδ on Y374 and Y374-PKCδ alters specific Ras-associated binding protein (RAB) lysosomal trafficking, which lessens RTK degradation and promotes RTK recycling [25]. Breast cancer cells with increased pY374-PKCδ levels (regulated by the opposing actions of FER and PTPN14) due to loss of PTPN14 accelerated the magnitude of ERK activation following ligand stimulation. Moreover, pY374-PKCδ inhibits Ras-associated binding protein 5 (RAB5) from late endosomes and compromises cargo degradation carried by the endosomes [25]. It was also confirmed that elevated pY374-PKCδ levels (induced by PTPN14 deficiency) led to increased cell surface expression of IGF1R and MET, as seen in EGFR tumour cells [25] (Fig. 1b). To expand the role of PKCδ in EGFR TKI resistance [20], it is tempting to hypothesise the relevance of Y374-PKCδ phosphorylation status in EGFR-mutant NSCLC cells regarding FER and PTPN14, as noted in breast cancer cells [25]. The Goodall group's discoveries [24, 25] on dysregulation of the FER-PKCδ-PTPN14 axis and high levels of RAB5-RAB7-positive transitional endosomes in triple-negative and HER2-positive breast cancers show that phosphorylation of Y374-PKCδ stabilises the transient RAB5-RAB7-positive endosome population to shift the balance from RTK degradation to recycling. FER over-expression correlated with poor post-operative survival in NSCLC [26], FER levels are elevated in ovarian cancer cells and loss of FER impaired the development of metastasis in ovarian cancer cells in vivo. FER phosphorylates MET at Y1349 with the activation of Src-homology 2 domain-containing phosphatase 2-mitogen-activated protein kinase (SHP2-MAPK) and RAC1-p21-activated protein kinase (RAC1-PAK) [27].
kinase (PAK1) signalling downstream of MET in ovarian cancer cells [27]. Patients with high PAK1 mRNA or low E-cadherin mRNA respond less to TKI than patients with low PAK1 or E-cadherin tumours. It was shown that miR-145 transcription is de-repressed by phosphoinositide 3-kinase (PI3K)/Akt-mediated C/EBP-B phosphorylation, increasing PAK1 expression in gefitinib-resistant LUAD cells [28]. We carried out similar experiments in HCC827 (EGFR 19 deletion) targeting PKCδ-PAK1 signalling pathways. The combination of auranofin plus OTSSP167 (MEK inhibitor) shows high synergism for inhibiting cell viability and colony formation. Mechanistically, the combination abrogates the expression of EGFR, MET, PAK1, PKCδ, ERK1/2, Akt, YAP1 and mTOR. The primary targets involved in: Cell-cycle progression, Proliferation, Survival, Downstream oncogenic signalling pathways, Activation of PLCγ, δ and receptor tyrosine kinase recycling. a PKCδ is a common mediator involved in EGFR TKI resistance. EGFR TKIs, like gefitinib, does not inhibit EGFR pY1173 and induce the formation of inactive EGFR heterodimers. The sustained pY1173 by EGFR heterodimer promotes the activation of PLCγ2 and PKCδ and downstream oncogenic signalling pathways. EGFR interacts with other RTKs, which are implicated in PKCδ and downstream oncogenic signalling pathway activation. b Increased pY374-PKCδ levels (regulated by the opposing actions of FER and PTPN14) increase the amount of RTKs on the cell surface. EGFR epidermal growth factor receptor, TKI tyrosine kinase inhibitor, Y tyrosine, p phosphorylation, PLCγ2 phospholipase γ2, PKCδ protein kinase Cδ, SHP2 Src-homology 2 domain-containing phosphatase 2, FER feline sarcoma-related, RAB5 Ras-associated binding protein 5, RTK receptor tyrosine kinase, CDCP1 CUB domain-containing protein [30]. Multiple tumour suppressors preserve the integrity of the Hippo pathway with cytoplasmic YAP retention, among them PTPN14 [30]. Mutations in neurofibromin 2 (NF2) have been noted in EGFR TKI-resistant NSCLC. NF2 triggers phosphorylation of YAP at S127, thereby causing cytoplasmic retention of YAP, thus preventing YAP from activating transcription of target genes. NF2 deletions have been identified by NGS in 3% of osimertinib-treated patients [17]. Upon tyrosine phosphorylation, YAP can also be activated by the Src family kinase (SKF), Yes [31] (see below). Activation of the YAP and FOXM1 axis as a driver of epithelial-to-mesenchymal transition (EMT)-associated EGFR TKI resistance was discovered through an integrated transcriptomic, proteomic, and drug screening approach. Nilsson et al. (Heymach’s group) [32] found that FOXM1 is a direct transcriptional target induced by YAP. The FOXM1 protein decreased in EGFR TKI-resistant cells treated with small interfering RNA targeting YAP. Pharmacological inhibition of YAP with CA3 or verteporfin diminished FOXM1 and spindle assembly checkpoint (SAC) components, such as Aurora A, Aurora B, polo-like kinase (PLK1), and kinesin spindle protein (KSP) in erlotinib resistant cells. Along with increased nuclear localisation of YAP, EGFR TKI-resistant cells have greater sensitivity to YAP inhibition with CA3 than with the parental cells. YAP induced EGFR TKI resistance to both gefitinib and osimertinib through up-regulation of AXL [6]. In addition, AXL is often over-expressed in EGFR TKI-resistant cells and associated with mesenchymal traits, such as loss of E-cadherin and vimentin expression in several PC9 gefitinib-resistant lines [15]. Interestingly, Nilsson et al. [32] noticed that EGFR TKI-resistant cells have increased abundance of AXL, but were not sensitive to the inhibition of AXL alone, or in combination with EGFR TKIs. The Heymach group’s discoveries [32] indicate that the YAP/FOXM1 transcriptional programme up-regulates SAC components. EGFR TKI-resistant cells are highly sensitive to the inhibition of both the transcriptional pathway and SAC components (KSP, PLK1 and survivin) (Fig. 3). In addition, EGFR TKI-resistant cells were sensitive to KSP inhibitors, such as ispinesib, with IC50 values in the low nanomolar range. On the same lines, aneuploid cancer cells show increased sensitivity to genetic perturbations of core components of SAC, which ensures the proper segregation of chromosomes during mitosis. It was recently reported that aneuploid cancer cells become increasingly sensitive to inhibition of SAC over time. Aneuploid cancer cells were vulnerable to depletion of a specific mitotic kinesin, KIF8A [33, 34]. It is plausible to assume that EGFR TKI-resistant cells are aneuploid. 5-phase kinase-associated protein 2 (SKP2) serves as an E3 ubiquitin-ligase repressing several proteins.
including p27 and p21. YAP promotes polyplody via Akt signalling, inducing Skp2 cytosolic retention [35]. Skp2 expression is highly up-regulated in cancers. Imipramine blue (an anti-depressant derivative) was shown to inhibit breast cancer growth by interacting and inhibiting FOXM1 and, subsequently, its transcriptional targets, including Skp2, PLK, and Aurora A, among others [36]. Furthermore, EGFR TKI-resistant cells are sensitive to Aurora A and Aurora B inhibitors [32, 37, 38]. Nanoparticle-based formulation of the Aurora B inhibitor, AZD2811, has the potential to increase efficacy and tolerability. Nilsson et al. [32] used The Cancer Genome Atlas data to assess the correlation between FOXM1 expression and SAC component gene expression in LUADs. In patients with EGFR-mutant NSCLC, FOXM1 expression was correlated with expression of PLK1 expression, AURKA/B (encoding Aurora kinase A and B), KIF11 (kinesin family member 11) (encoding KSP) and BIRC5 (baculoviral IAP repeat-containing 5) (encoding survivin) (Fig. 3). High FOXM1 expression was associated with worse disease-free survival in EGFR-mutant NSCLC. In addition, FOXM1 also enhances the activation of HGF/MET signalling extracellular signal-regulated kinase 1/2, phosphoinositide 3-kinase/Akt and signal transducer and activation of transcription 3 (STAT3) in pancreatic ductal adenocarcinoma [39] (Fig. 3). N6-methyladenosine (m6A) mRNA modification regulates mRNA splicing, export, stability and translation. ALKBH5 (α-ketoglutarate-dependent dioxygenase homologue 5) is an RNA m6 demethylase elevated in glioblastoma stem-like cells that regulates FOXM1 (Fig. 3). The long-chain non-coding RNA (lncRNA) antisense to FOXM1 promotes the interaction of ALKBH5 with FOXM1 nascent RNA, which favours demethylation and elevated expression of FOXM1. It was shown that FOXM1 is a central mediator in glioblastoma stem-like cell proliferation [40]. Moreover, FOXM1 can act downstream of STAT3 in a ferritin-STAT3-FOXM1 feedback loop. It is noted that FOXM1 expression is observed across all subtypes of glioblastoma multiforme, while phosphorylated STAT3 is more restricted to mesenchymal subtype and absent in pro-neural glioblastoma multiforme [40].

**Targeting YAP-mediated tumour-lineage plasticity**

Plasticity between different signalling pathways, adaptive activation of bypass signalling, is inherent in temporal tumour heterogeneity under therapeutic selective pressure [41]. See the differential STAT3 phosphorylation at S727 and Y705 regulating EMT and MET processes [42] (see below). YAP/TEAD engages the EMT transcription factorSlug to directly repress pro-apoptotic BMF. In PC9 (EGFR-mutant cells) standalone osimertinib leads to re-colonisation of wells within 8 weeks. In the combination of osimertinib with trametinib (MEK inhibitor), a few viable cells are still detected after 15 weeks of treatment. However, within days following drug withdrawal, cells proliferate and re-colonise the wells. Higher YAP/TEAD activity was observed, as measured by connective tissue growth factor (CTGF) in osimertinib-induced dormant (non-proliferative) PC9 cells. EGFR-mutant NSCLC cell lines treated for 3 weeks with osimertinib plus a tankyrase inhibitor (XAV939) reduced the number of dormant cells, diminishing regrowth. The study adds further evidence that YAP activation is necessary for cancer viability upon combined EGFR/MEK inhibition. YAP inhibition by XAV939 increases BMF (a pro-apoptotic BH3-only protein) expression in response to osimertinib in EGFR-mutant NSCLC cell lines in vitro and in vivo [43]. It was previously reported that tankyrase and the canonical Wnt pathway protect EGFR-mutant lung cancer cells from EGFR inhibition [44].

Activation of the Hippo pathway is triggered by a core kinase cascade that contains the mammalian sterile 20-like kinases (MST1/2), the large tumour suppressor 1/2 (LATS1/2) and the scaffolding protein Salvador (Sav1) (Fig. 2). Sequential phosphorylation-dependent cytoplasmic retention of the transcriptional coactivator YAP and its paralogue TAZ (transcriptional coactivator with PDZ-binding motif) occurs through interaction with 14-3-3, while the retained YAP/TAZ is downregulated by β-Trcp-mediated proteolytic degradation (Fig. 2). The regulation of YAP/TAZ subcellular localisation occurs through a variety of upstream regulators, such as Merlin, Kibra and Angiomotin, that
physically bind to and exclude YAP/TAZ from the nucleus. Merlin binds to Angiomotin, releasing Rich1 (a small GTPase-activating protein) to inactivate RAC1-GTP to RAC1-GDP. In response to growth stimuli, Merlin dissociates from Angiomotin. Unoccupied Angiomotin binds to and blocks Rich1’s GAP activity, leading to increased levels of Rac1-GTP [45]. Also, PTPN14 induces cell-density-dependent nuclear export of YAP, thereby suppressing YAP oncogenic function. Mastermind-like (MAML) is a coactivator of Notch-dependent transcription that also promotes Wnt, Shh and NF-κB signalling target gene transcription. It has recently been shown that MAML1/2 also induces nuclear localisation of YAP/TAZ and enhances YAP/TAZ-TEAD-mediated transcriptional activity via the formation of a functional complex. Tissue microarray shows a strong correlation between MAML2 and YAP levels in lung cancer patients. The Cancer Genome Atlas (TCGA) data reveal a strong correlation between the mRNA levels of MAML2 and YAP target genes in lung cancer [46]. In addition, actionable YAP1-MML2 fusions have been identified in glioblastoma (AM-38), ovarian cancer (ES-2) and head and neck carcinoma (SAS) cell lines. The fusion brings together exons 1–5 of YAP1 and exons 2–5 of MAML2. YAP1-MML2 fusions are associated with increased YAP1 signalling [47]. Mir-30c contains a highly conserved region targeting MAML1 mRNA, establishing the mir-30c-MAML1-YAP/TAZ axis, which should be considered for further research [46]. Several microRNAs (miRNAs), IncRNAs and circular RNAs interact with YAP and numerous key components of the Hippo pathway [48]. Zanconato et al. [49] determined that YAP/TAZ physically engages the general coactivator bromodomain-containing protein 4 (BRD4). BET inhibitors impaired the expression of YAP target direct genes, such as AXL, AURKA, FST1 and PDL1 [49]. The relevance of the YAP/TAZ-BRD4 connection in EGFR-mutant NSCLC warrants analysis.

The regulation of cytoplasmic sequestration of YAP/TAZ has also been associated with Hippo-independent mechanisms where YAP is activated by the SFK, Yes, upon tyrosine phosphorylation. The seminal work of Karin’s group [31] in inflammatory bowel diseases and colorectal cancer showed that gp130, a co-receptor for interleukin-6 (IL-6) cytokines, triggers activation of YAP and Notch, independently of the gp130 effector STAT3 [31]. The data indicate that activation of gp130 contributes to healing, regeneration and termination of inflammation via SFK-YAP-Notch signalling, in addition to the effect of STAT3 [31]. Previously, we found that gefitinib or osimertinib activates STAT3 and Src-YAP1 in EGFR-
EGFR-mutant NSCLC patients to test safety and response rate study of osimertinib plus repotrectinib (formerly, TPX-0005) in Figure 3 epitomises our conceptualisation of the interplay between RTKs and SFKs-YAP1 [6]. We have initiated a phase I individual clinical evolution in an EGFR-mutant NSCLC patient is histological change, developing a spindle-like morphology asso-

Hippo pathway and resistance to chemotherapy

TKI-resistant EGFR-mutant cells (i.e. H1975 resistant) undergo a histological change, developing a spindle-like morphology associated with a shift of E-cadherin expression loss and appearance of vimentin, indicating EMT phenotype. An early description of individual clinical evolution in an EGFR-mutant NSCLC patient is represented by a patient with EGFR L858 LUAD and a TP53 mutation with a discreet response to first-line chemotherapy. The patient achieved near-complete response to her lung metastasis with erlotinib. After 8 months of erlotinib, a progression of lung metastases occurred. A lung core biopsy revealed the presence of EGFR T790M mutation. There was no response to chemotherapy and the patient developed bone and liver metastasis. However, a second lung core biopsy revealed the L858R EGFR mutation, but no detectable T790M mutation [53]. Compared with gefitinib alone, gefitinib combined with carboplatin plus pemetrexed improved PFS and OS, but with increased toxicity [54, 55]. Lysosomal sequestration of cytotoxic drugs and targeted therapies has been described [56]. Low lysosomal pH causes drug sequestration, impeding the release of cytotoxic agents and/or targeted agents to the cytoplasm. The effect has been reverted by verteporfin through a YAP-independent mechanism [56]. It has been reported that the Hippo-YAP pathway is involved in gemcitabine activity. Nuclear YAP enhances gemcitabine effec-

Mutations in the Hippo-YAP pathway are present in association with loss of tumor suppressor genes, including INK4a/ARF and PTEN, which are commonly lost in many human tumors. The Hippo-YAP pathway is an important regulator of cell proliferation, energy metabolism, and cell growth. In cancer cells, the Hippo-YAP pathway is often activated, leading to increased cell proliferation and decreased apoptosis. Overactivation of the Hippo-YAP pathway can lead to tumor progression and metastasis. Therefore, targeting the Hippo-YAP pathway may be a potential strategy for cancer therapy.

In the presented study, the authors investigated the effects of gemcitabine on EGFR-mutant NSCLC cells in vitro and in vivo. They found that gemcitabine was able to inhibit cell proliferation and induce apoptosis in EGFR-mutant NSCLC cells. In addition, they observed that gemcitabine was able to downregulate the expression of the EGFR pathway components, including Akt and the MAPK pathway. Furthermore, they found that gemcitabine treatment was associated with a reduction in YAP1 expression, which is an important downstream effector of the Hippo-YAP pathway. These results suggest that gemcitabine may have the potential to inhibit the Hippo-YAP pathway in EGFR-mutant NSCLC cells.

The authors also investigated the in vivo efficacy of gemcitabine in a xenograft model of EGFR-mutant NSCLC. They found that gemcitabine treatment was able to inhibit tumor growth and prolong survival in EGFR-mutant NSCLC xenografts. These results suggest that gemcitabine may be a promising therapeutic agent for the treatment of EGFR-mutant NSCLC.

In conclusion, the present study demonstrates the potential of gemcitabine to inhibit the Hippo-YAP pathway and to induce apoptosis in EGFR-mutant NSCLC cells. These findings suggest that gemcitabine may be a promising therapeutic agent for the treatment of EGFR-mutant NSCLC. Further studies are needed to evaluate the efficacy and safety of gemcitabine in clinical trials.
change >1.2, 15 genes were up-regulated in EGFR-mutant tumours in the TCGA cohort. The top up-regulated gene was ADORA1 (up-regulation fold change = 2.16, P < 0.001) encoding the adenosine receptor 1, and the sixth up-regulated gene was NT5E (up-regulation fold change = 1.44, P = 0.02) encoding CD73, both of which are key molecules in the CD73/adenosine pathway. An ongoing trial combining oleclumab (anti-CD73) with osimertinib or olceumab and AZD4635 (adenosine receptor inhibitor) (NCT03381274) evaluates the CD73/adenosine pathway blockade in EGFR-mutant NSCLC [63]. It was seen that inhibition of ADORA1 induces the up-regulation of PD-L1 via transcription factor ATF3 in melanoma and NSCLC. ADORA1 and ATF3 levels predicted the efficacy of PD-1 immune checkpoint inhibitors [64]. Ataxiatelangiectasia mutated (ATM)-associated DNA-damage-induced T cell senescence and dysfunction by both regulatory T cells and tumour cells. The study indicates that ATM-associated DNA-damage initiation and MAPK and STAT3 signalling activation control lipid metabolism in senescent T cells in vitro. Blockade of ATM activation with the ATM-specific inhibitor, KU55933, decreased mRNA and protein expression of group IV phospholipase A2α in regulatory T-induced senescent T cells [65].

Neurotransmitters in EGFR-mutant NSCLC

Monique Nilsson and John Heymach [66] reported that stress hormones (norepinephrine) activate β2-adrenergic receptors on NSCLC cells that cooperate with mutant EGFR, resulting in the inactivation of LKB1 and subsequent secretion of IL-6. β2-Adrenergic (ADRB2) activation, with tumour growth and EGFR TKI resistance, was abrogated with β-blockers or IL-6 inhibitors. Propranolol (β-blocker) blocked norepinephrine-induced IL-6. We found synergism with carvedilol (another β-blocker in clinical practice), but not with propranolol in EGFR-mutant resistant cells pretreated with norepinephrine (unpublished). mRNA expression of ADRB2 was elevated in NSCLC patients, as well as in NSCLC cell lines. Elevated IL-6 determined by enzyme-linked immunosorbent assay (ELISA) was associated with worse prognosis in EGFR TKI-treated NSCLC patients, while β-blocker use was associated with lower IL-6 concentrations and better benefit from EGFR TKIs [66]. Recently, it has been reported that a gut microbe formed by the gut microbiota called Presumably multi-phenylacetyl glutamine that activates TrkA activates TrkB and STAT3 signalling, both in cancer cell lines and in experimental mice models [67]. Presumably, multi-facorial stimuli can converge in up-regulating platelets in experimental mice models [67]. Apparently, multi-facorial stimuli can converge in up-regulating platelets in experimental mice models [67].

STAT3, IL-6 and leukaemia inhibitory factor (LIF)

STAT3 is a latent transcription factor found in the cytoplasm and is activated by tyrosine phosphorylation, leading to dimerisation and nuclear translocation [77]. We adopted immunofluorescence to test STAT3 activation state by its intracellular localisation. STAT3 was activated and translocated into the nucleus with gefitinib, but this did not occur with TPX-0005 and the combination [6]. STAT3 Y705 phosphorylation occurs a few hours after treatment with EGFR TKIs inducing surviving tumour cells to adapt to a quiescent, dormant persistent state [78]. We noted that afatinib equally causes phosphorylation of STAT3 Y705 and elevation of STAT3 and RANTES (regulated upon activation, normal T cell expressed and presumably secreted) mRNA levels. Aldehyde dehydrogenase-positive cells were observed and HE51 (Hairy and Enhancer of Split 1) was elevated following therapy with afatinib plus TPCA-1 (STAT3 inhibitor) [79]. As previously observed [77], we depicted a model showing that EGFR-mutant lung cancer cells produce high IL-6 levels, which subsequently activates the gp130/JAK/STAT3 pathway. Unphosphorylated STAT3 (U-STAT3) activates genes, such as RANTES, when induced by the activation of STAT3 in response to EGFR mutations or ligands (i.e. IL-6). Although afatinib inhibits ERK and Akt signalling in PC9, PC9-GR2 (AXL over-expression) and PC9-GR4 (38% allelic fraction of T790M), it does not abolish phosphorylation of STAT3 Y705. Also, a gradual increase in STAT3 and RANTEs mRNA levels was observed after 7 days of treatment. Jacqueline Bromberg’s group [77] examined mRNA levels of IL-6, OSM (oncostatin M), LIF, IL-11 and CNTF (ciliary neurotrophic factor), but only IL-6-mRNA was detected in 11–18, H3255, H1650 and H1975 cell lines. IL-6-secreted protein levels were determined by ELISA in the conditioned medium collected for near-confluent cell cultures: 11–18 cells, 1800 pg/ml; H3255 cells, 2900 pg/ml; H1650 cells, 7700 pg/ml; and H1975 cells, 7800 pg/ml. These levels were higher in comparison with levels of normal cells or other cancer-derived cell lines for which no >10–60 pg/ml were seen [77]. These data, together with other experiments, helped to discover that IL-6 is secreted by EGFR-mutant NSCLC cells, leading to the activation of the gp130/JAK/STAT3 signalling pathway [77].

LIF receptor (LIFR) is a well-recognised upstream regulator of the Hippo pathway. LIFR expression suppresses nuclear YAP disposition in breast cancer cells. LIFR expression status correlates with PFS in breast cancer patients [80]. Intriguingly, LIF activates LIFR/p-ERK pathway in breast cancer cells. LIFR expression correlates with PFS in breast cancer patients [80]. Intriguingly, LIF activates LIFR/p-ERK/STAT3 S727 phosphorylation in MET process in lung cancer. The findings provide a new glimpse of the putative processes in EMT/MET in EGFR-mutant NSCLC. By studying metastasis in bone marrow-derived mesenchymal stem cell (BM-MSC)-driven lung cancer models, it was shown that BM-MSCs elicit EMT in epithelial-type cells through the IL-6/STAT3 Y705 phosphorylation pathway while inducing MET in mesenchymal-type cells through LIFR/STAT3 S727 phosphorylation [42]. These findings complement the Bromberg group’s seminal discoveries [77]. STAT3 phosphorylation at Y705 and S727 is a readout of EMT and MET fluctuations according to the tumour evolution and adaptive mechanisms of treatment resistance. Such a concept warrants further analysis in EGFR-mutant NSCLC and could provide a new means for gauging the plasticity of EGFR-mutant cancer cells. It is tempting to speculate that the levels of IL-6 and LIF could be surrogate markers in the evolution of EGFR-mutant NSCLC.

Further convolution in EGFR TKI resistance (tolerance) is the role of SOX2 and TGFβ signalling [81–84]. As aforementioned, TKI
therapy favoured mesenchymal traits in lung cancer cells, with deficient SOX2 expression, whereas SOX2 expression promotes TKI sensitivity and inhibited the mesenchymal phenotype [81]. SOX2 belongs to the SOX (Syr-related HMG Box) family of proteins and responds to respiratory tract injuries. SOX2 signalling initiates the proliferation and differentiation of lung progenitor cells to maintain tissue homeostasis. SOX2, in conjunction with OCT4, KLF4 and MYC, can reverse the mesenchymal morphology of fibroblasts and reprogramme them into pluripotent stem cells. Inhibition of TGB signalling facilitates the SOX2-mediated reprogramming process of fibroblasts [81]. Tumours expressing low SOX2 and high vimentin signature were associated with worse survival outcomes in EGFR-mutant patients [81]. Moreover, TGFBR1/2 receptors were up-regulated in HCC827 GR and H1975 AZD-R-resistant lines. Epigenetic modifications of H3K27ac and H3K4m3 (which marks the active enhancer and promoter, respectively) were higher in the SOX2 locus in HCC827 cells compared with its TKI-tolerant counterpart, HCC827 GR. The study clearly shows that the loss of SOX2 expression by TGFβ switches off SOX2-EGRF signalling and induces EMT with decreased BCL2L11 (Bim) pro-apoptotic signalling; henceforth, increasing EGRF TKI tolerance [81].

**Concurrent alterations in EGFR-mutant NSCLC**

Targeted NGS performed by Helena Yu and investigators at Memorial Sloan-Kettering Cancer Center in 200 EGFR-mutant pretreatment samples revealed that the most frequent concomitant genetic defects were mutations in TP53, PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha), CTNB1 and RB1, and focal amplifications in ETRF, TTF1, MDM2, CDK4 and FOXA1. Moreover, shorter PFS with EGFR TKI was related to the amplification of ERBB2 or MET, or mutations in TP53. In post-treatment samples, in addition to EGFR T790M (51%), MET (7%), BRAF fusion, FGFR3 fusion, YES amplification and KAEI was also detected [85]. Other primary studies showed the bonafide of outlining co-existing genetic defects in EGFR-mutant NSCLC using circulating tumour DNA (ctDNA)-targeted NGS assays [86]. The co-occurrence of TP53 mutations is high, 65–75%, in ctDNA or tumour samples, respectively, and correlates with shorter PFSI [87–90].

Patients with EGFR/RB1/TP53-mutant NSCLC represented 5% (43 of 863) of EGFR-mutant NSCLC and, irrespective of the risk of small cell lung cancer transformation, patients with EGFR/TP53/ RB1 mutations had a shorter time to discontinuation than EGFR/ TP53 and EGFR-mutant only cancers (9.5 versus 12.3 versus 36.6 months, respectively) [90]. Arsenic trioxide (ATO) used for the treatment of acute promyelocytic leukaemia can rescue multiple p53 mutants. ATO inhibits the growth of cell lines with p53 mutations at structural hotspots in five lines: RXF 393 (R175H), CCRF-CEM (R175H R248Q), Hop-92 (R175L), Sk-MEL-2 (245S) and BT-549 (R249S). ATO did not significantly reactivate any DNA-contact p53 mutants. In contrast, the structural p53 mutations, V272M, R282W, E285K and V234C, were rescued by ATO in transactivation. This study highlights the opportunity of repurposing ATO in treating p53-mutant cancer patients [91].

Patients with concurrent EGFR/TP53/RB1 alterations also displayed gene defects in PIK3CA (20%), NTRK1 (11%), MCL1 (11%), NK2 homeobox 1 (11%), ERBB2 (9%), FOXA1 (9%), PCLY2 (9%), PTEF (9%), RNA-binding motif protein 10 (RBM10) (9%) and others [90]. Activation-induced cytokine deaminase/apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like APOBEC mutation was also enriched in EGFR/TP53/RB1-mutant NSCLC [90]. Intriguingly, APOBEC3B (A3B) cytosine deaminase is repressed by p53, while p53 mutation increases A3B expression. In addition, cytotoxic agents such as cisplatin, etoposide or 5-fluorouracil induced A3B expression and cytotoxic deamination. Chemotherapy induction of A3B expression is directed by DNA-PKcs/ATM/Akt activation of NF-kB. In T47D cells, the DNA-PKcs inhibitor, NU7411, inhibited A3B expression in a dose-dependent manner. In all cancer cell lines tested, chemotherapy drugs stimulated A3B expression and DNA-PK, ATM, Akt or NF-κB inhibitors reduced cisplatin-induced A3B expression [92].

**FUTURE PERSPECTIVES**

LUAD driven by EGFR mutations is one of the most recognised and studied types of non-small cell lung cancer, specifically at the research level in human EGFR-mutant cell lines, and at the clinical level in pharmaceutical industry-oriented clinical trials. This review stresses basic key points accumulated in laboratory research, with the goal of making the information accessible and translatable to EGFR-mutant patients, both as novel biomarkers and as combinatory therapies for improving survival in stage IV EGFR-mutant LUAD patients. NGS assays are indubitably necessary for managing EGFR-mutant guidance; however, at present, co-mutational landscape information is merely informative since no therapies are currently available for patients with commonly co-occurring TP53 mutations. In the authors’ opinion, further endeavours should be made to yield patient-derived organoids, as they could offer a wide opportunity to better understand the cancer cell dynamics of each individual patient beyond the standardisation of care.

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