

A Complex Disease: Non-Small Cell Lung Cancer

Subjects: Pathology

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Non-small cell lung cancer (NSCLC) is a complex disease often driven by activating mutations or amplification of the epidermal growth factor receptor (EGFR) gene, which expresses a transmembrane receptor tyrosine kinase. Targeted anti-EGFR treatments include small-molecule tyrosine kinase inhibitors (TKIs), among which gefitinib and erlotinib are the best studied, and their function more often imaged. TKIs block EGFR activation, inducing apoptosis in cancer cells addicted to EGFR signals.

Keywords: Non-small cell lung cancer ; epidermal growth factor receptor ; egfr ; fluorescence ; microscopy

1. A Brief Outline of Non-Small Cell Lung Cancer

Lung cancers are classified in two main histological groups: small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) ^[1]. SCLC comprises ~15–20% of all primary lung tumours and is often caused by smoking ^[2]. NSCLC frequently arises among non-smokers and can be sub-divided into adenocarcinoma, squamous cell carcinoma, the most prevalent, large cell carcinoma, and bronchial carcinoid tumour ^[3]. Like all tumours, dysregulated cell division is driven in NSCLC by genetic alterations, the accumulation of which eventually enables tumour cells to acquire limitless replicative potential ^[4]. Gene sequencing technologies have allowed the identification of driver oncogenic gene alterations in the *EGFR* gene itself ^[5], and/or of genes expressing oncogenic proteins within EGFR's downstream signalling pathways, especially those that regulate cell survival and proliferation, on which tumour initiation and growth critically depend ^[6] (examples in [Box 1](#)). Mutations in genes downstream of *EGFR* decouple cell growth and proliferation from EGFR signalling, hence anti-EGFR drugs become ineffective.

Box 1. Some Common Oncogenes in NSCLC.

EGFR	EGFR is one of the four members of the human epidermal growth factor (HER) family transmembrane receptors (HER1/EGFR, HER2, HER3, and HER4). The prevalence of <i>EGFR</i> oncogene mutations is 50% among Asian patients with lung adenocarcinoma and 15% among Western patients ^[7] . Exon 19 deletions or L858R point mutations in exon 21 account for 90% of the activating mutations in the tyrosine kinase domain of EGFR, resulting in constitutive activation of EGFR without growth factor-induced stimulation, thus promoting cell proliferation ^[8] .
KRAS	KRAS is the predominantly mutated RAS isoform (85%) and also the most frequent oncogene in NSCLC ^[9] . KRAS fosters tumour growth via several mechanisms, including by upregulating rate-limiting enzymes involved in amino acid, fatty acid, or nucleotide biosynthesis, and by stimulating scavenging pathways, such as macropinocytosis and autophagy ^{[9][10]} , which, in turn provide building blocks for the anabolic routes, also maintaining the energy levels and the cell's redox potential ^[11] .
BRAF	BRAF is a proto-oncogene encoding a serine-threonine protein kinase acting downstream of the RAS/RAF/ERK signalling pathway. BRAF carries signals from membrane receptors (such as EGFR) to the nucleus of the cell to regulate DNA transcription ^[12] . BRAF is an oncogene located on chromosome 7 involved in several cell functions, including growth, proliferation, survival, and differentiation. Immunotherapy is beginning to show promise as an active therapy in BRAF -mutated NSCLC ^[13] .
ALK	The ALK gene encodes the ALK tyrosine kinase receptor and is associated with many types of cancers, including NSCLC ^[14] . There are three types of ALK mutations: rearrangement (ALK-R), amplification (ALK-A), and point mutation. ALK gene rearrangement is a driving mutation underlying the development of NSCLC ^[15] , which appears to be more common in younger patients and never or light smokers diagnosed with adenocarcinoma. ALK can phosphorylate STAT3 and PI3K independently of ERK to antagonise apoptosis and promote cell survival ^[16] .
TP53	The TP53 gene encodes a DNA damage check point p53 protein, which is at the heart of the cellular decision to proliferate or activate programmed cell death. It regulates the transcription of ~500 genes ^[17] , including cell cycle regulatory genes and transcription factors, and DNA repair genes ^[18] . Over 50% of human cancers carry loss of function mutations in TP53 , with the mutant form acting as a dominant-negative inhibitor towards the wild-type moiety. When chromosomal abnormalities or environment stresses become overwhelming, p53 can arrest cell-cycle progression and induce apoptosis. TP53 alterations carry a worse prognosis in NSCLC ^[19] .

MYC

A family of three human proto-oncogenes (*c-MYC*, *I-MYC*, and *n-MYC*) code for transcription factors [20]. In normal cells, depending on nucleotide pools' levels, growth signals, glucose, or oxygenation, elevated MYC expression can cause apoptosis. Transformed cells can, however, adapt to constitutively elevated levels of MYC expression, resist its apoptotic effects, and only respond to MYC pro-proliferative signals either via loss of growth suppression surveillance mechanisms (e.g., *TP53* mutation) and/or by gain of pro-survival signals. *MYC* is a metastasis gene for NSCLC [21].

Surgery, radiation, and chemotherapy remain among the first-line treatments for NSCLC [22]. More targeted therapies include immune check-point inhibitors, engineered cytotoxic chimeric antigen receptor-immune T cells, oncolytic viruses, anti-tumour vaccines, and small-molecule inhibitors against oncogenes driving NSCLC tumours [23]. Of interest here is the sub-class of quinazoline-derived small-molecule EGFR-selective tyrosine kinase inhibitors (TKIs) that target EGFR, and specifically first-generation gefitinib and erlotinib, because these two TKIs are still commonly employed as first-line therapies [24] and have also been extensively investigated via fluorescence microscopy methods.

EGFR signalling is at the heart of cell growth and proliferation. This makes EGFR mutations highly susceptible to be exploited by cancer cells to alter their physiology and achieve immortalisation [5]. Key oncogenetic *EGFR* alterations upon which NSCLC tumours become addicted to EGFR signals include somatic mutations in the *EGFR* gene clustered around the periphery of the catalytic adenosine triphosphate (ATP)-binding cleft in EGFR's kinase domain (**Figure 1A**). The two most common are a point substitution in exon 21 (L858R), which, for example, accounts for 90% of all NSCLC activating oncogenic EGFR mutations in the NSCLC Caucasian patient subset, and an in-frame deletion in exon 19 (e.g., DL746-P750) [25] (**Figure 1B**). Lower-frequency mutations include point mutations in exon 18 (G719X, G719S, G719A) and exon 20 (V765A and T783A) [26]. Different mutations can display different sensitivities to TKI inhibition of autophosphorylation and downstream signals (see, for example, [27]).

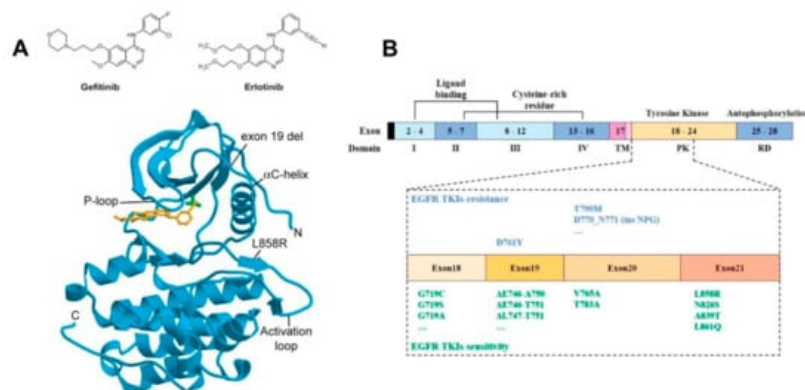


Figure 1. Gefitinib and erlotinib binding to EGFR's kinase domain. **(A)** Top: Structures of gefitinib and erlotinib; bottom: Schematic representation of the wild-type EGFR tyrosine kinase domain (cyan) bound to erlotinib (orange) (PDB entry 1M17). The threonine 790 side chain is shown in green (top right of the bound TKI). EGFR numbering includes the 24 residue signal sequence [28]. Conserved structural features essential to the activation of the kinase domain, the phosphate-binding loop (P-loop), the α C-helix, and the activation loop are shown. Sites of common NSCLC TKI-sensitive mutations (exon 19 deletion and L858R substitution) are also shown. Reproduced from [28]. **(B)** Schematic representation of the domains of EGFR and the corresponding exons. Specific NSCLC-related mutations in the kinase domain of EGFR (exons 18–24) that are associated with sensitivity or resistance to EGFR-TKIs are denoted [29]. Reproduced from [29].

The first tumour-suppressing responses to TKI therapy were observed for gefitinib and erlotinib almost 20 years ago (see, for example, [30][31][32]). Orally administered, these TKIs reversibly outcompete the binding of ATP to the phosphate-binding loop in the kinase domain of EGFR, thus suppressing its tyrosine kinase activity [33] (**Figure 1A**). A striking response was found in a subset of ~10–40% of patients who harboured NSCLC tumours driven by somatic activating mutations in the first 4 exons of the tyrosine kinase domain of the *EGFR* gene [34][35] (**Figure 1B**). This success led to the approval of gefitinib and erlotinib for the treatment of NSCLC patients bearing such mutations [36].

Another common driver of NSCLC found in 50–90% of cases is an increase in the *EGFR* copy number, which often results in the overexpression of wild-type EGFR (wtEGFR) [37]. Among these tumours, approximately 80% were found to be de novo resistant to gefitinib and erlotinib despite TKIs potentially blocking the kinase activity and autophosphorylation of wtEGFR [38]. The intrinsic resistance of wtEGFR-expressing tumours to TKIs is recapitulated by many other solid tumour types [39]. This is so even in the absence of mutations in effectors downstream of EGFR that decouple growth and survival pathways from EGFR signalling [45] (**Box 1**). The reasons for this are not well understood.

APOPTOSIS: Associated plasma membrane structural changes include translocation of the anionic phosphatidyl serine (PS) from the inner to the outer leaflet of the lipid bilayer where it can bind Annexin V, a Ca^{2+} -dependent phospholipid-binding protein with high affinity for PS. By labelling Annexin V with fluorescent dyes (e.g., FITC [40]), one can image, for example, via wide-field or confocal microscopy, and/or flow cytometry, Annexin V-positive cells to determine the rate of apoptosis [41]. Fluorescent Annexin V conjugates provide a quick and reliable detection method of the early stages of apoptosis [42]. Apoptosis in cells can also be detected, for example, by imaging fluorescence conjugates of Bax as it translocates from the cytosol to the outer mitochondrial membrane, and/or cytochrome C as it is released from the mitochondria into the cytosol [43].

ENDOCYTOSIS: Immunostaining against endosomal protein markers [44] includes against early endosome proteins (Syntaxin 6 and Rab5 [45], and EEA1 [46]), recycling endosome markers (e.g., Rab25 [47]), and late endosome/lysosomal markers (Rab7 [48], LAMP1 and LAMP2 [49], cathepsin D, and LIMP2 [50]). Primary or secondary antibodies can be conjugated with dyes of different colours (e.g., Alexa 488, Alexa 594, or Alexa 647). Typical endosomes (~100 nm) are smaller than optical resolution (~250 nm), hence endosomes look like puncta under a wide-field or confocal fluorescence microscope. To image EGFR endocytic traffic, one can, for example, label an EGFR cognate ligand (e.g., EGF) with organic dyes, both visible and infrared [51], or clone EGFR with tags, such as Halo [52] and SNAP [53], which are subsequently labelled with Alexa or Cyanine dyes. In live cells, one can use fluorescent protein (FP) fusions of the endosomal markers and/or of other proteins (e.g., clathrin [54]). To image in the nucleus, a popular method is fluorescence in situ hybridisation (FISH) assays [55].

AUTOPHAGY: FP constructs of the 17 kDa soluble microtubule-associated protein 1A/1B-Light Chain 3 (LC3) [56] are commonly used (e.g., eGFP-LC3, mCherry-LC3, or RFP-LC3). During autophagy, the cytoplasmic form of LC3 (LC3-I) becomes covalently ligated to phosphatidyl ethanolamine (PE). The appearance of fluorescent puncta of the lipidated LC3-II form allow determination via wide-field or confocal fluorescence microscopy of the number of autophagosomes (dia. 500–900 nm [57]), where LC3-II is recruited to [58]. Serum depletion and the autophagic inhibitor 3-methyladenine (chloroquine) are often used as positive controls [59]. Colocalisation of red and green probes (e.g., RFP-LC3 and LysoSensor Green) allows for the morphological observation and quantification of autophagosome maturation and fusion with the lysosome [60]. pH-responsive FPs (and organic dyes) allow the evaluation of intracellular pH and interrogation of specific subcellular compartments [61].

SINGLE PARTICLE TRACKING (SPT): A direct probe of fluorescent particle movement in live cells [62]. In two colours, SPT can report molecular association and dissociation events in real time from which kinetic and dynamic interaction parameters can be determined (e.g., [63][64]). At the plasma membrane, SPT exploits total internal reflection fluorescence (TIRF) illumination to improve contrast [65]. Suitable organic dyes and other probes have to be selected to ensure specific interactions with the proteins of interest and to minimise non-specific staining of the (typically) glass surface where the TIRF evanescent wave illuminating the adjacent basolateral cell surface is concentrated [66][67]. SPT can also be used to track particles in endosomes and at the nucleus using probes, such as adaptamers and FPs, and/or bright organic dyes, such as Atto 647N (e.g., [68][69]).

NEAR-FIELD SCANNING OPTICAL MICROSCOPY (NSOM) [70]: The resolution of NSOM is defined by the size of the point light source used (typically 50–100 nm). NSOM breaks the far-field optical resolution limit (~250 nm) by exploiting the properties of evanescent waves in close vicinity (i.e., ~nanometres) of the aperture defining the size of the point light source, which must therefore be brought within nanometres of the surface to collect the near-field optical signal. The point source is scanned over the surface, without touching it. The distance between the point light source and the sample surface is usually controlled through a feedback mechanism that is unrelated to the NSOM signal (e.g., as in AFM) [71].

STOCHASTIC OPTICAL RECONSTRUCTION MICROSCOPY (STORM) [72]: A single-molecule localisation microscopy (SMLM) method with a resolution of ~20 nm. It reports on the number of proteins that form nanoclusters and on the size of the clusters (example shown in Figure 6). STORM is compatible with many commonly used organic dyes, which can be converted to an off state using specific excitation parameters combined with oxygen-scavenging imaging buffers. Fluorophores for STORM should be bright, have a high rate of photo-switching, and exhibit minimal photo-bleaching in thiol-containing buffers. Normally used to analyse clusters in chemically fixed cells, sub-12 nm resolution is possible in cryo-vitrified samples using solid immersion lenses [73].

FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET): A spectroscopic ruler useful for measuring intra-molecular and inter-molecular separations in the range ~2–8 nm [74]. It is based on the transfer of excitation energy between two fluorescent molecules through non-radiative dipole–dipole coupling [75][76]. The rate of energy transfer, from which the separation between donor and acceptor molecules can be measured, is determined chiefly from the overlap

between the emission spectra of the donor and the excitation spectra of the acceptor. FRET can be combined with SPT [77][78][79] (Figure 6E), fluorescence lifetime imaging (FLIM) [80], and fluorescence polarisation [81]. The combination can be used to detect dimers and oligomers, and/or to determine separations between two planes, as a proxy for molecular orientation at the plasma membrane [82].

FLUOROPHORE LOCALISATION IMAGING WITH PHOTOBLEACHING (FLImP) [83][84]: Based on SMLM, the position of a cluster of fluorescent molecules changes upon each individual photobleaching event. The shift in the position of the cluster can be analysed to report on the lateral separations between the molecules in the cluster. FLImP can measure separations between identical fluorophores in the 0–60 nm range, and can achieve sub-5 nm resolution [85]. Combined with atomic molecular dynamics (MD) simulations, it can report on the dimer and oligomer structure [85][86].

2. EGFR's Role in the Development of NSCLC Tumours

2.1. EGFR Structure and Signalling Pathways

EGFR is the founding member of the family of four human receptor tyrosine kinases (HER1–4) [87]. Additionally termed ErbB1 because of its close similarity with the avian viral v-erb-B oncogene protein [88], EGFR was cloned and sequenced in the early 1980s [89], and is ubiquitously expressed in epithelial, mesenchymal, and neuronal cells [90]. Structurally, the EGFR consists of a growth factor-binding ectodomain made out of four subdomains, a single-pass transmembrane alpha helix, an inner juxtamembrane segment, a kinase domain locus of EGFR's intrinsic protein tyrosine kinase activity, and a long unstructured C-terminal domain [91] (Figure 2A). EGFR binds seven cognate growth factors, namely epidermal growth factor (EGF), transforming growth factor alpha, betacellulin, heparin-binding EGF-like growth factor, epiregulin, and epigen [92]. Growth factor binding induces a conformational change in EGFR's ectodomain [93] that exposes a loop required for ectodomain dimerization [94][95] (Figure 2A). This leads to allosteric changes across the plasma membrane, chiefly the formation of a catalytically active asymmetric kinase domain dimer [96], via which EGFR becomes phosphorylated (p-EGFR) in five key C-terminal tyrosine phosphorylation sites (Tyr992, Tyr1045, Tyr1068, Tyr1086, and Tyr1173) [97][98]. Activating EGFR mutations and EGFR overexpression elicits growth factor-independent constitutive receptor dimerisation and/or oligomerisation, thereby activating the catalytic activity of the receptor without the need for the growth factor stimulus [86][99]. This allows EGFR to trigger downstream signalling pathways in a growth factor-independent dysregulated fashion, ultimately eliciting uncontrolled cell division and tumour proliferation [5][38].

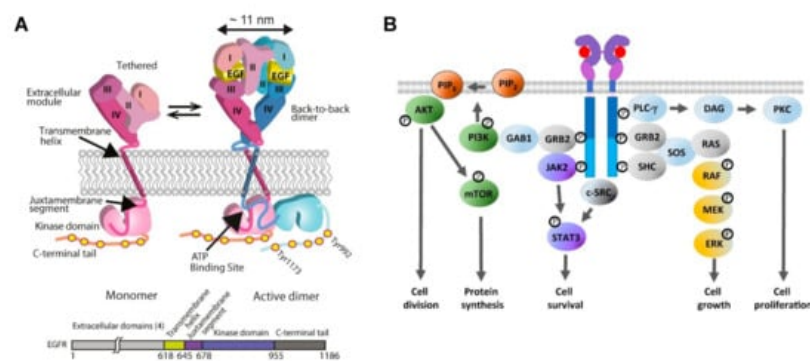


Figure 2. (A) Cartoon of the EGF-induced receptor dimerisation process and an EGFR sequence diagram. Left: A tethered single-pass EGFR monomer [93]. Right: The EGFR monomer binds EGF to form an extended back-to-back ectodomain dimer [94][95], structurally coupled via an N-crossing dimer of two transmembrane alpha-helices [99] to an asymmetric tyrosine kinase dimer [96], in which the activator kinase (pink) allosterically activates a receiver kinase (blue), which phosphorylates the C-terminal domain of the donor kinase [96][99]. Reproduced from [85]. (B) Growth factor-dependent EGFR signalling pathways. EGFR activates the RAS/extracellular signal-regulated kinase (ERK) pathway for cell growth, and the JAK/signal transducer and activator of transcription 3 (STAT3) signalling cascade for cell survival. Activation of the PI3K/AKT/mammalian target of rapamycin (mTOR) signalling pathway leads to cell division via AKT phosphorylation and protein synthesis via mTOR phosphorylation. EGFR activates Phospholipase C gamma (PLCγ), which in turn activates the PKC signalling pathway, leading to cell proliferation [100].

Summarised [100] in Figure 2B, EGFR recruits via its C-terminal pY992 the Src Homology 2 (SH2) domain of PLC-γ, which hydrolyses PIP₂, releasing diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP₃), and leading to the activation of PKC and cell proliferation. EGFR can recruit via pY1068, pY1148, and pY1173 the SH2/SH3 adaptors GRB2 and SHC, which bind via their SH3 domains the protein scaffolds SOS and GAB1 to initiate well-defined tyrosine/serine/threonine phosphorylation cascades [101]. One is the RAS-RAF-MEK-ERK1/2 signalling pathway, which leads to ERK activation and translocation of ERK from the cytoplasm to the nucleus, where it upregulates genes that promote cell growth [102]. GRB2

also recruits via GAB1 the lipid kinase PI3K [103]. PI3K catalyses PIP₂ into PIP₃, which recruits AKT, leading to the activation of the PI3K-AKT-mTOR signalling pathway. Phosphorylation of AKT leads to the inhibition of antagonists of Cyclin D1 and cell division [104]. AKT-mediated phosphorylation of mTOR upregulates the cell's anabolic metabolism [105]. Phosphorylated EGFR also activates the JAK2/STAT3 signalling axis to upregulate the transcription of a variety of proteins involved in the survival of cancer cells [106]. EGFR also interacts with c-SRC, a crucial non-receptor tyrosine kinase and an oncogenic partner in EGFR-driven NSCLC [107]. Among many other pro-survival functions [108], c-SRC synergises with EGFR to activate STAT3 in a JAK-independent manner [109].

2.2. TKI Treatments Induce Apoptosis via the Mitochondrial Intrinsic Pathway

The oncogenic addiction of some NSCLC tumours to dysregulated EGFR signalling underpins the rationale for treating the disease by using TKIs to stop the p-EGFR-dependent downstream signalling pathways that are essential to sustain uncontrolled cell proliferation, thereby inducing programmed cell death [110]. Early experiments in lung adenocarcinoma A549 cells [111] showed that termination of p-EGFR signals by gefitinib resulted in phosphorylation and activation of the cell cycle regulator protein p53 (Box 1), followed by p53-dependent upregulation of PUMA, a pro-apoptotic, BCL2 homology 3 (BH3) domain-containing member of the BCL2 family [112], which activates rapid induction of the caspase-dependent intrinsic apoptosis pathway [113] (Figure 3). Gefitinib also upregulated pro-apoptotic Fas and downregulated the anti-apoptotic proteins survivin and XIAP [111]. Further experiments in TKI-sensitive lung adenocarcinoma cell lines (PC-9 and H1560, which express the D746–750 deletion EGFR mutant, and H1975 that express the L858R mutant) showed that erlotinib dramatically induces the expression of BIM, another pro-apoptotic BH3-only member of the BCL2 family [112], which, like PUMA, also mediates TKI-induced apoptosis via the intrinsic pathway of caspase activation [114] (for a transcriptional profiling of NSCLC cell lines, see [115][116]). In cells with activating EGFR somatic mutations, BIM's pro-apoptotic effects are synergistic with the loss of survivin, whose downregulation enhances gefitinib-induced apoptotic death in TKI-sensitive NSCLC cells [117]. These results were confirmed in lung tumours and xenografts from mice bearing mutant EGFR-dependent lung adenocarcinomas, which also display increased concentrations of BIM after erlotinib treatment [127]. Gefitinib and erlotinib also block EGFR phosphorylation of ERK and AKT, therefore pushing the closely regulated equilibrium maintained by the BH3-only BCL2 family towards the activation of effector members BAK and BAX, which thereby form oligomers at the outer mitochondrial membrane, leading to mitochondrial outer membrane permeabilisation (MOMP) and apoptosis [118][119] (Figure 3). Confocal microscopy images in live cells of the cellular distribution of BAX fused to GFP before and 3 h after stimulating apoptosis via treatment with staurosporine [120] are also shown in Figure 3.

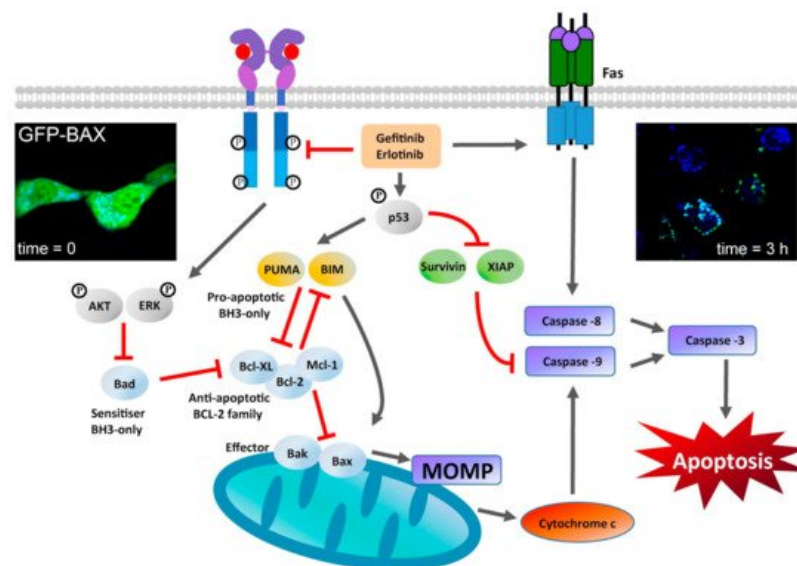


Figure 3. Stimulation of the mitochondrial-dependent intrinsic apoptosis pathway by gefitinib and erlotinib. This pathway is marked by a key event—mitochondrial outer membrane permeabilization (MOMP)—which results in the release of cytochrome c from the mitochondrial intermembrane space. MOMP can be triggered by the activation of BH3-only proteins of the BCL-2 family [112] following their post-translational modification (e.g., phosphorylation) [121]. Activated BH3-only proteins generally stimulate MOMP by inducing the oligomerization of BCL2-associated X protein (BAX) and/or BCL2 antagonist or killer (BAK) in the outer mitochondrial membrane, thereby forming supramolecular channels that mediate the liberation of cytochrome c [118]. At the cytosol, cytochrome c triggers the assembly of a caspase-activating complex between caspase 9 and apoptotic protease-activating factor 1 [122]. TKI inhibition can stimulate the transactivation of genes encoding pro-apoptotic proteins (such as the BH3-only protein p53-upregulated modulator of apoptosis (PUMA)). Gefitinib and erlotinib can also activate the so-called death receptor FAS, leading to activation of caspase 8 [111][112].

Caspase 8 proteolytically activates downstream effector caspases or truncates the BH3-only protein BID (BH3-interacting domain death agonist), which co-activates the intrinsic pathway of apoptosis by translocating to mitochondria. Caspase-8 interacts with caspase-9 to activate the executioner caspase-3, which coordinates the destruction of cellular structures, such as DNA fragmentation or degradation of cytoskeletal proteins ^[123]. Fluorescence image inserts: left: Live cell image of exogenous GFP-BAX expressed in D407 cells (immortalized human retinal pigment epithelial cells); right: same area imaged after inducing apoptosis using 1 μ M staurosporine prepared in DMSO. Images were taken using a spinning disk confocal microscope, which is ideal for fast 3D imaging of live cells and using an EM-CCD camera. Scale bar = 5 μ m. Images reproduced from ^[120].

2.3. The Development of Resistance to TKI Treatment

Even among the NSCLC patients that respond, the effects of gefitinib and erlotinib are transient (mean progression-free survival of 10–14 months) ^[124]. Approximately 50% of NSCLC cases develop a secondary point substitution in exon 20 of the *EGFR* gene (T790M), which confers resistance to first-generation TKI by impeding the inhibition of receptor phosphorylation through a substantially increased affinity of the *EGFR*'s kinase domain pocket for ATP ^[125]. New generations of TKIs have been developed in a race to overcome the effects of the single T790M and double L858R/T790M mutations, including second-generation (irreversible) afatinib and dacomitinib, and third-generation (T790M selective) osimertinib, which are currently used in the clinic ^[126]. However, further mutations in the *EGFR* gene and of downstream effectors eventually allow tumours to overcome the TKI therapeutic block and resume uncontrolled proliferation ^{[6][127]}.

Acquired mutations not only involve the *EGFR* gene (e.g., the secondary T790M acquired *EGFR* mutation and others ^[26]), but can also be *EGFR* independent (e.g., loss of p53 function, constitutive activation of RAS, etc. ([Box 1](#))). Together, acquired mutations contribute to increase tumour heterogeneity and develop pro-survival adaptation mechanisms at cellular and tumour levels ^{[128][129]}. However, for such mutations to accumulate, cells need to first survive the initial therapeutic insult. An important observation is that TKIs fail to trigger apoptosis in a fraction of responsive NSCLC tumour cells addicted to *EGFR* signals, instead inducing G1 cycle arrest ^[27]. Whilst the latter contributes to suppress tumour growth, quiescent cells surviving TKI treatment have the opportunity to acquire mutations and/or invoke adaptation mechanisms by which they can eventually resume uncontrolled proliferation. Chiefly among *EGFR*-dependent mechanisms of adaptation are pro-survival functions exercised by *EGFR* independently of its kinase activity, which can be recapitulated in quiescent cells by the actions of TKI-bound *EGFR*s ^[39]. Examples of *EGFR* kinase-independent functions include stimulation of DNA synthesis ^[130], expression of the *c-fos* proto-oncogene ^[131], and dysregulation of cellular self-degradation processes ^[132], with the latter extensively imaged by fluorescence microscopy methods.

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