Drug Resistance Mechanisms in Non-Small Cell Lung Carcinoma

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Abstract: Lung cancer is the most commonly diagnosed cancer in the world. "Driver" and "passenger" mutations identified in lung cancer indicate that genetics play a major role in the development of the disease, progression, metastasis and response to therapy. Survival rates for lung cancer treatment have remained stagnant at ~15% over the past 40 years in patients with disseminated disease despite advances in surgical techniques, radiotherapy and chemotherapy. Resistance to therapy; either intrinsic or acquired has been a major hindrance to treatment leading to great interest in studies seeking to understand and overcome resistance. Genetic information gained from molecular analyses has been critical in identifying druggable targets and tumor profiles that may be predictors of therapeutic response and mediators of resistance. Mutated or overexpressed epidermal growth factor receptor (EGFR) and translocations in the echinoderm microtubule-associated protein-like 4 (*EML4*)-anaplastic lymphoma kinase (*ALK*) genes (EML4-ALK) are examples of genetic aberrations resulting in targeted therapies for both localized and metastatic disease. Positive clinical responses have been noted in patients harboring these genetic mutations when treated with targeted therapies compared to patients lacking these mutations. Resistance is nonetheless a major factor contributing to the failure of targeted agents and standard cytotoxic agents. In this review, we examine molecular mechanisms that are potential drivers of resistance in non-small cell lung carcinoma, the most frequently diagnosed form of lung cancer. The mechanisms addressed include resistance to molecular targeted therapies as well as conventional chemotherapeutics through the activity of multidrug resistance proteins.

Keywords: Non-small cell lung cancer, EGFR, EML4-ALK, tyrosine kinase inhibitors, drug resistance, ABC transporters, ABCB1, ABCC1, ABCC10, ABCG2.

INTRODUCTION

Lung cancer is a significant health concern in the United States as it is the leading cause of cancer related deaths. Worldwide, it is also the most commonly diagnosed cancer, the primary cause of cancer related deaths for men and is surpassed only by breast cancer in women as a cause of cancer related deaths [1, 2]. Tobacco usage has long been the major avoidable behavioral factor linked to lung cancer and is the cause of ~30% of all cancer deaths in developed countries [3]. Voluntary and non-voluntary (second hand smoke) contributes to ~90% of lung cancer cases [4, 5]. Tobacco induced carcinogenesis occurs through various mechanisms including formation of bulky DNA adducts [4], induction of inflammation [6], increased oxidative stress [7] and activation of diverse signaling pathways [6]. Furthermore, environmental and occupational respiratory carcinogens such as asbestos may interact with cigarette smoke and increase the probability of developing lung cancer [8].

Genetic susceptibility also appears to play a role in lung cancer especially in people that develop the disease at 50 years of age or less, compared to people who develop the disease within the median age of onset (66 years) [9]. Familial aggregation of lung cancer has also been reported. Specifically, having a parent or a sibling diagnosed with lung cancer increases the relative risk of developing the disease by 1.5 to 6.1 fold in both smokers and non-smokers, respectively [10, 11]. In addition, several high risk genes that predispose non-smokers to lung cancer have been identified [12, 13]. Studies are required to determine the association between these susceptibility genes and clinical outcomes.

Non-small cell lung cancer (NSCLC) is the most commonly diagnosed form of the disease accounting for over 85% of the cases [14]. NSCLC is a heterogeneous aggregate of histologies with the most common being adenocarcinoma, large cell carcinoma and squamous cell (epidermoid) carcinoma [15, 16]. These histologies have different clinical characteristics but share similar treatment approaches and prognoses. Adenocarcinoma is the most common histology indicated in 35-40% of the cases and most prevalent in non-smokers [15, 17].

The 5 year survival rate of NSCLC varies depending on stage at diagnosis from 52.2% to 25.1% to 3.7% in patients with local, regional or advanced metastatic disease respectively [18, 19]. Approximately, 20-30% of patients present with stage I, II and IIIA disease which is treated with curative intent by surgical resection, chemotherapy and radiotherapy [20, 21]. Stage IA patients have 5 year survival rates of 73% while 5 year survival rates for stage IIIA patients are

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24% [20, 22, 23]. Adjuvant cisplatin based therapy increases these survival rates by 5% but disease recurrence is a major issue [24]. However, there is no way to predict patient response as 21% of stage IA and 42% of stage IB disease patients recur and die of the disease while 42% of Stage II patients are cured by surgery and do not require cisplatin adjuvant chemotherapy [20, 22, 25].

Approximately 40-50% of NSCLC patients present with advanced or metastatic stage IIIB and IV disease, are not candidates for curative therapy and are treated with systemic chemotherapy [19, 21]. For patients with unresectable stage III disease, combination platinum-based chemotherapy and radiotherapy is recommended, whereas in stage IV disease, chemotherapy alone is customary [26, 27]. Among these patients, systemic therapy is the only treatment proven to increase survival by up to 8-12 months, decrease symptoms and improve quality of life [28, 29]. The choice of treatment for patients with advanced disease is dependent on the histological subtype of the disease, molecular tumor characteristics, performance status, comorbidities and prior therapy [29, 30]. In metastatic disease, treatment is based on the combination of cisplatin or carboplatin with drugs such as paclitaxel, docetaxel, gemcitabine and vinorelbine which exhibit superior efficacy compared to single agent platinum therapy [28, 29]. Toxicity appears to be the only major difference between the various platinum-based doublet regimens as they offer similar clinical benefits [31].

Molecular targeted therapies are now included in the treatment regimen of NSCLC since they have been shown to extend progression free survival and improve overall survival [32-34]. The presence of molecular biomarkers such as mutations or amplification of epidermal growth factor receptor (EGFR) [34, 35], echinoderm microtubule-associated protein-like 4-naplastic lymphoma kinase (EML4-ALK) kinase translocation [36], mutations in KRAS [37] and PI3KCA [38] can serve as targets for these therapies but also as indicators of treatment outcomes.

Similar to conventional chemotherapies, these newer targeted agents also have a propensity to fail due to the development of resistance. Drug resistance which can be either pre-existent (intrinsic) or acquired after drug exposure is a major cause of failure in the treatment of malignant disease and remains an unsolved pharmacological problem [39, 40]. Chemoresistance is regulated by complex mechanisms which through multiple cell adaptations, render drugs ineffective in cell killing [41]. Although research has identified some of the adaptations, how to reverse this issue in NSCLC is still enigmatic. As a result, there have been numerous attempts to overcome drug resistance in order to improve the efficacy of chemotherapy and understanding these mechanisms will be critical in solving the resistance issue. In this review, we examine mechanisms through which NSCLC becomes resistant to both targeted therapies and conventional chemotherapeutic agents.

RESISTANCE TO TARGETED AGENTS IN NSCLC

Epidermal Growth Factor Receptor (EGFR)

Epidermal growth factor receptor (EGFR, HER-1, ERBB1) is a member of the epidermal growth factor receptor tyrosine kinase family which consists of 3 additional receptors with similar structure: EGFR2/HER-2-NEU/ERBB2, EGFR3/HER-3/ERBB3 and HER4/ERBB4 [42, 43]. These receptors are expressed primarily on the surface of epithelial cells and contain an extracellular ligand-binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain that mediates signal transduction [44, 45]. Upon receptor binding, the receptors form homo or heterodimers, the receptor-ligand complex gets internalized and auto-phosphorylation on tyrosine residues occurs resulting in activation of downstream signaling cascades [46]. Erbb receptors have roles in normal development as Egfr knockout mice die *in utero* and exhibit gross abnormalities of the brain, heart, bone and other epithelial organs [47, 48]. These receptors are implicated in the development and progression of cancer due to their ability to modulate cell cycle progression, apoptosis, cell migration, angiogenesis, migration and drug resistance [49].

Research has shown that EGFR plays an important role in the growth, survival and chemoresistance in NSCLC either by aberrant expression or mutation. Overexpressed EGFR has been reported in 40-80% of NSCLC [45, 50]. Overexpression can occur as a result of various mechanisms including an increase in gene copy number, epigenetic modifications and activation by oncogenic viruses [51, 52]. Somatic activating mutations in the EGFR tyrosine kinase domain (exon 18-21) and deletions of exon 19 have been identified in 10-15% of Caucasian patients and 30-40% of Asian patients [53]. The overexpression or constitutive mutation of EGFR leads to the activation of various signal cascades including the phosphatidylinositol 3-

kinase/AKT pathway (PI3K/AKT), the mitogen activated protein kinase pathway (MAPK) and the signal transducers and activators of transcription (STAT) pathway [54, 55]. EGFR overexpression correlates with disease progression, decreased survival, lymph node metastasis and poor chemo-sensitivity [56, 57].

In the past two decades, a variety of tyrosine kinase inhibitors (TKIs) targeting EGFR have been tested in clinical trials. First generation TKIs such as erlotinib and gefitinib inhibit EGFR tyrosine phosphorylation through competitive, reversible binding to the ATP site on the kinase domain [34, 58]. In large randomized studies, erlotinib as a second or third line therapy was shown to confer a survival advantage [59] while gefitinib did not demonstrate a survival advantage except in select clinical subgroups of Asians and never-smokers [60]. Monoclonal anti-EGFR antibodies such as cetuximab, directed to the extracellular domain of the receptor also have reported clinical benefit [56, 61]. In randomized studies comparing the addition of cetuximab to first line chemotherapy, patients with high EGFR expression demonstrated increased overall survival with no meaningful side effects [56, 61].

Among patients with EGFR activating mutations, 70% respond to TKI treatment, while the remaining 30% show intrinsic resistance to these inhibitors [62, 63]. Among patients with intrinsic resistance, presence of drug resistant mutations and modifications in EGFR signaling are well studied mechanisms [63]. The missense mutation in exon 21 (L858R) and the in-frame deletion in exon 19 are more sensitive to TKIs than the exon 20 (T790M) mutation [54]. Interestingly, T790M germ line mutations have been identified in a European family with genetic susceptibility to

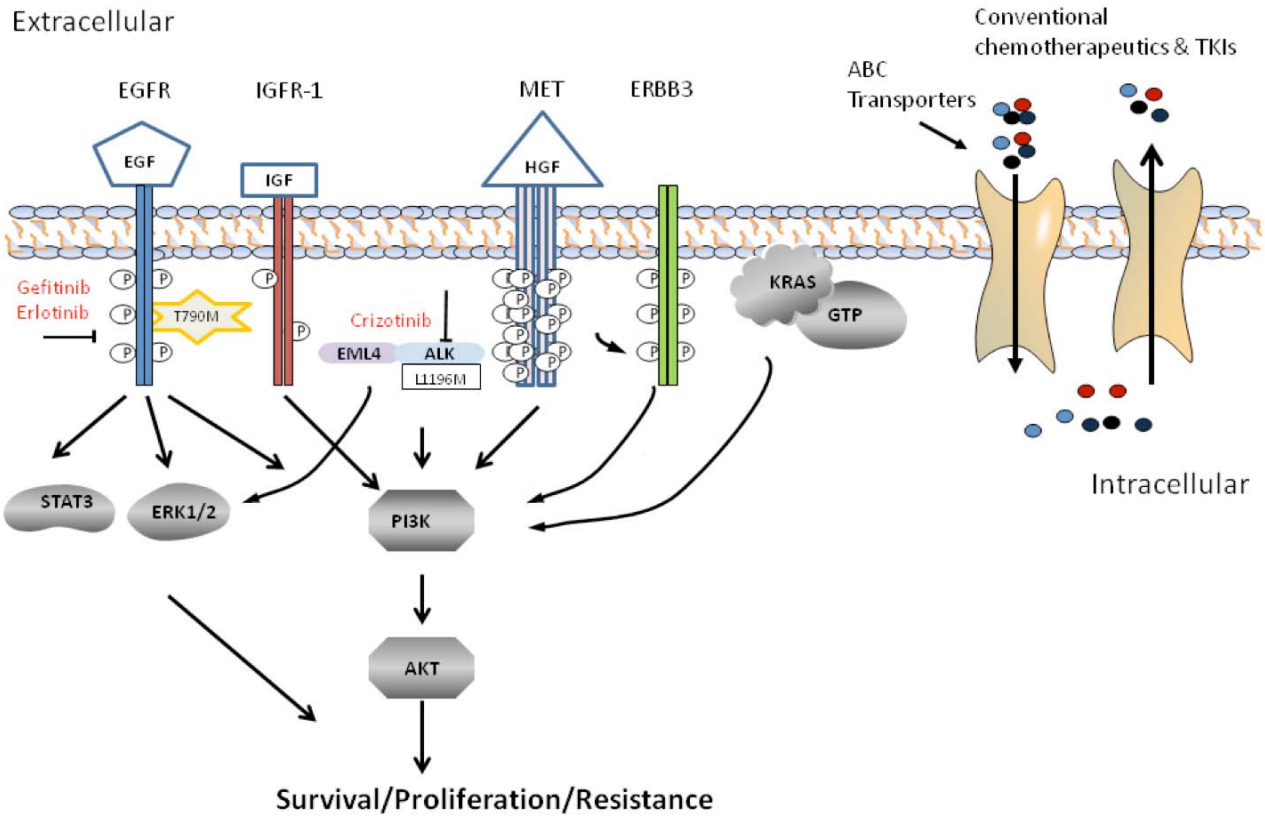

Figure 1: A simplified illustration of mechanisms of resistance to conventional therapeutics and TKIs in NSCLC. Response to TKIs targeting epidermal growth factor receptor (EGFR) can be affected by mechanisms that include the development of drug insensitive secondary mutations (T790M) in the kinase domain of the receptor; crosstalk with insulin-like Growth Factor Receptor 1 (IGFR-1); cross talk of amplified *MET* with epidermal growth factor receptor 3 (ERBB3) and mutations in KRAS. These mechanisms result in activation of signaling effectors such as STAT3, ERK1/2 and PI3K/AKT which support the proliferation and survival of drug resistant cells. ABC transporters such as ABCG2 and ABCB1 have been shown to efflux both TKIs and conventional therapeutics. The resulting decrease in intracellular drug concentrations is a factor in drug resistance. Targeting the oncogenic fusion protein echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase (EML4-ALK) with TKIs such as crizotinib has also been shown to be negatively affected by the development of secondary gate keeper residue mutations such as L1196M, and the emergence of fusion negative tumors that render the disease insensitive to the drug. EGF (epidermal growth factor), IGF (insulin-like growth factor, HGF (hepatocyte growth factor), GTP (guanosine triphosphate).

bronchioalveolar carcinoma, implicating EGFR signaling in lung cancer susceptibility [64]. In sporadic lung cancer with no exposure to tyrosine kinase inhibitors, the mutation has been detected, albeit at very low frequency [65]. In NSCLC cells and tumors treated with tyrosine kinase inhibitors, this mutation has been shown to be one of the major determinants and causes of drug resistance [65, 66].

Increased PI3K/AKT Signaling

Intrinsic resistance to EGFR inhibitors is associated with increased signaling through the phosphatidylinositol 3-kinase (PI3K)/AKT pathway as a consequence of *PTEN* loss [67, 68]. In erlotinib resistant H1650 lung cancer cells, genomic loss of *PTEN* was accompanied by high levels of phosphorylated AKT [67]. Rescue of *PTEN* loss through expression of exogenous *PTEN* resensitized the cells to erlotinib. In addition, analysis of tumor biopsy samples showed enrichment of EGFR mutant samples with hemizygous loss of chromosome 10 on which *PTEN* is located [67]. The loss of *PTEN* may induce resistance in patients with EGFR mutations possibly by relieving the tumor's dependence on EGFR signaling [67, 69]. Mutations in the PI3KCA and P110α subunits of PI3K can also induce primary resistance to EGFR TKIs through constitutive AKT activation [70].

Insulin-Like Growth Factor Receptor 1 (IGFR-1) Crosstalk

Crosstalk of EGFR with the Insulin like Growth Factor Receptor 1 (IGFR-1) can also induce intrinsic resistance to EGFR targeted therapies. In a study of surgically resected patients, high co-expression of EGFR with IGFR-1 was reported to have a poor prognosis and was associated with decreased survival [71]. The activation of IGFR-1 by binding of IGF-I and IGF-II to its extracellular domain results in the activation of both MAPK and PI3K/AKT pathways. It is through the activation of PI3/AKT pathway that IGFR-1 can promote resistance to EGFR targeted therapies [72]. An enriched pool of pre-TKI resistant cells that depend on IGFR-1 signaling have been discovered in a heterogeneous pool of cancer cells and only an IGFR-1 inhibitor could prevent the emergence and expansion of EGFR TKI resistant cells [73].

KRAS Mutations

Mutations of the *KRAS* proto-oncogene appear to have a negative effect on NSCLC treated with EGFR TKIs [74-76]. These mutations have been observed in 15-25% of lung cancer samples and in particular, 30-50% of adenocarcinomas [74, 77]. These mutations are found mutually exclusive to EGFR activating mutations as KRAS is a downstream effector of the receptor, making these tumors unresponsive to upstream EGFR inhibition therapy [78]. The most commonly observed mutations in KRAS are point mutations on codons 12, 13 and 61 which appear to be an early irreversible event in lung tumorigenesis [79, 80]. KRAS possesses intrinsic GTPase activity which when constitutively activated by mutations can result in cell transformation and unregulated growth [79, 81]. KRAS mutations may also be linked to smoking as they are more common in smokers while EGFR mutations are commonly observed in never smokers [74, 82, 83]. However, KRAS mutations have also been found in cases of never smoker patients indicating that despite the strong correlation between smoking and these mutations, predicting KRAS mutation status should not depend primarily on a patient's smoking history [37].

Early studies on the role of KRAS mutations on EGFR targeted therapy yielded conflicting information. Some studies showed that in resected NSCLC, KRAS was a negative prognostic factor while others showed no value of KRAS mutations in prognosis [77, 84, 85]. A meta analysis performed by Mascaux *et al*., with results from 53 clinical studies pointed to KRAS as a negative prognostic factor in adenocarcinomas [86]. In NSCLC patients treated with erlotinib or gefitinib, lack of clinical response was common in patients with KRAS mutations [87, 88]. KRAS may be used to predict chemotherapy outcomes as mutations decrease the response rates of patients treated with cytotoxic drugs such as docetaxel, paclitaxel and carboplatin [76, 89]. In the clinic, the presence of KRAS mutations could be used to prevent the administration of chemotherapy to patients who are unlikely to benefit. This information may establish a potential rationale for prospective KRAS mutation testing before clinical trial or therapy assignment. Some promising data has also recently emerged showing that the MEK kinase inhibitor selumetinib may benefit KRAS mutant patients who have failed prior therapy [90].

Secondary Mutations in Acquired Resistance

Acquired resistance to EGFR inhibitors is mediated by the development of secondary mutations in the kinase domain of exon 20 at the gatekeeper residue T790M [65, 66]. This gatekeeper residue is a conserved threonine residue located in the hinge region of the kinase domain at the back of the ATP-

binding pocket and controls access to a hydrophobic sub-pocket [91]. This mutation has been found in over 50% of patients who were initially responsive to TKI therapy and it is hypothesized that clones in the tumor population carrying this mutation are selected for with TKI therapy [53, 66, 92]. This gatekeeper residue is important as it controls access to the hydrophobic pocket. Mutations change the conformation of the receptor, blocking binding of the TKIs, increasing both the enzymatic activity of the protein and the affinity of mutant proteins for ATP [93-95]. This T790M mutation is analogous to the gatekeeper residue T3151 in BCR-ABL kinase that is associated with acquired resistance to gleevec and imatinib in chronic myelogenous leukemia [96].

Other secondary mutations have been identified but they occur at lower frequency than the T790M mutation [66, 97]. They also confer a lesser degree of resistance than the T790M mutation [98]. These mutations include the T854A located on exon 21 [99], L747S and D761Y both on exon 19 [97, 98]. The T854A mutation was identified in patient samples treated with TKIs but not in their pre-treatment samples [99]. The residue is not conserved in other tyrosine kinases and is located at the bottom of the ATP binding pocket. The mutation causes a conformation change that prevents contact with the TKI. The D761Y mutation is located on the α-C-helix of EGFR and is similar to a mutation in BCR-ABL, D276G, that is associated with acquired resistance after imatinib treatment [100]. The L747S mutation lies at the start of the β3 strand and the α-C-helix [98] and has analogous residues in BCR-ABL (L237M) and ERBB2 (L755S or P) that have been identified in TKI resistant disease [98].

The occurrence of these secondary mutations spurred development of second and third generation irreversible TKIs which bind covalently to the cysteinyl-797 residue in the pocket of the EGFR-kinase domain and overcome resistance driven by the T790M mutation [101]. These agents show greater potency in the inhibition of kinase activity *in vitro* and *in vivo* compared to erlotinib and are in various stages of clinical testing [102, 103]. Dacotinib appears promising in phase I and II clinical trials in NSCLC that has failed TKIs and chemotherapy. It shows benefit in progression free survival and a trend towards increased overall survival [102]. Afatinib, an EGFR and HER2 irreversible inhibitor has been shown to reverse the effects of the T790M mutation in preclinical lung cancer studies [104]. In clinical trials, afatinib did not improve overall survival but showed a modest improvement in progression free survival after failure of erlotinib or gefitinib [105, 106].

Several drawbacks are associated with these irreversible EGFR-TKIs. The covalent attachment of the kinase inhibitors to the kinase pocket does not discriminate against wild type EGFR or the mutant proteins [94]. This may be the primary reason why these drugs modestly inhibit mutant T790M driven cancer signaling and suggests that these TKIs are less effective than previously thought. Recently however, a PEG-ylated anilinoquinazoline derivative labeled with ^{18}fludeoxyglucose (^{18}F) developed for use in positron emission tomography (PET) imaging has shown the ability to discriminate between wild type EGFR, mutant L858R or T790M in the irreversible binding which is of benefit in a clinical setting [107]. Dose limiting toxicities are another significant issue with these irreversible TKIs. Excessive adverse effects such as severe diarrhea result in reduction in drug dosage and interruptions in treatment which lowers the bioavailability of the inhibitors to levels insufficient to suppress EGFR activity [108]. Thus, further studies are required to determine dosing schedules that alleviate the adverse effects in order to achieve clinically effective inhibitor levels.

Epithelial to Mesenchymal Transition

In cancer cells, epithelial to mesenchymal transition (EMT) a process characterized by the loss of epithelial junction markers such as E-cadherin or γ-catenin and gain of mesenchymal markers such as fibronectin or vimentin is associated with progression, metastasis and drug resistance [109, 110]. In NSCLC cells and tumors, an EMT signature of 76 genes has been identified by Byers *et al*., through high throughput genomic and proteomic analysis in the quest to understand how this transition affects the disease [111]. It has been reported that EMT decreases sensitivity to conventional chemotherapeutics or inhibitors of EGFR and PI3K *in vitro*, *in vivo* and in patients [110, 112]. Decreased expression of E-cadherin and increased expression of fibronectin or vimentin appears to be a common feature of NSCLC cells and tumors resistant to gefitinib or erlotinib regardless of EGFR status and independent of secondary mutations [113]. Restoration of the epithelial phenotype by E-cadherin expression or inhibition of Axl, Notch and TGF-β [114] signaling has been shown to restore drug sensitivity and should be explored as alternate therapeutic strategies in lung cancer.

MET Amplification

Overexpression of hepatocyte growth factor (HGF) receptor through amplification of the *MET* gene has been observed in both TKI naïve and treated patients [115-117]. Amplified *MET* has been implicated in TKI resistance and has been detected in ~20% of patients exhibiting acquired resistance to EGFR inhibitors [117]. Resistance from *MET* amplification occurs as a result of aberrant activation of PI3K signaling *via* epidermal growth factor receptor 3 (ERBB3) driven mechanisms [115]. Monoclonal MET and HGF antibodies as well as small molecule kinase inhibitors are in various stages of preclinical and clinical trials [117, 118]. Combinatorial therapies with EGFR and *MET* inhibitors may be beneficial in alleviating resistance in lung cancer patients.

Echinoderm Microtubule-Associated Protein-Like 4-Anaplastic Lymphoma Kinase (EML4-ALK)

The *EML4-ALK* fusion gene is formed after the fusion of the echinoderm microtubule-associated protein-like 4 *(*EML4*)* and anaplastic lymphoma kinase *(*ALK*)* [36, 119]. ALK is a transmembrane protein which is not normally expressed in the lung [36], while EML4 is a ubiquitously expressed, intracellular localized protein with roles in microtubule assembly [120]. ALK has been previously identified in tumors showing chromosomal rearrangements such as anaplastic large cell lymphoma and inflammatory myofibroblastic tumors [121]. In lymphomas, ALK fuses with nucleophosmin (NPM) forming the NPM-ALK fusion product which drives survival of anaplastic large cell lymphoma [122]. The ALK fusion point appears conserved and results in fusion of the whole extracellular domain of ALK to its fusion partners [36]. This chimeric protein is constitutively active and can activate both MAPK and PI3K/AKT signaling [36]. Over 10 variants of EML4-ALK fusion products arise from different breakpoints on EML4 exons and all appear to have transformative abilities [123].

EML4-ALK appears uncommon in lung cancer and has been detected in about 3-8% of overall NSCLC cases [124, 125]. In never smokers, some studies indicate that the prevalence maybe as high as 20-30% of NSCLC cases [126, 127]. The clinical characteristics associated with EML4-ALK mutations are similar to those of EGFR with the translocation more commonly observed in never smokers with adenocarcinoma histology tumors who tend to be younger with a median age of 52 and have advanced stage disease [125, 127]. The translocation is found mutually exclusive of EGFR and KRAS mutations and predicts a poor response to EGFR TKI therapy [126, 127]. In a study by Shaw *et al*., patients with *EML4-ALK* translocations treated with erlotinib did not show a clinical response to erlotinib treatment and had lower progression free survival as well as overall survival [127]. In the same study, patients with the translocation treated with standard cytotoxic therapies showed a lower response rate to platinum therapies compared to patients with EGFR mutations though these differences were not statistically significant [127]. These findings are highly similar to those observed in patients with KRAS mutations. Thus it appears that NSCLC with *EML4-ALK* translocation needs to be treated as distinct subgroup with its own pathological and clinical features which would benefit from ALK targeted therapy.

Various ALK inhibitors have been tested pre-clinically and clinically with crizotinib showing the most promise [128]. Crizotinib is an oral ATP competitive tyrosine kinase phosphorylation inhibitor that can inhibit both ALK and MET kinases [129]. A phase I clinical trial of crizotinib reported an impressive response rate of 57% with progression free survival of over 6 months by study reporting time and minimal toxicity [124]. In a follow up phase II trial, crizotinib treatment was associated with increased overall survival in advanced NSCLC when given as a second or a third line agent [130].

EML4-ALK Secondary Mutations

Similar to other tyrosine kinase inhibitors, patients treated with crizotinib eventually develop resistance and mutations that lead to resistance have been reported [131, 132]. In one report, the patient developed two *de novo* mutations within the kinase domain of EML4-ALK, C1156Y and L1196M [131]. The L1196M mutation has been shown to be a “gatekeeper” mutation similar to that observed in NPM-ALK that also renders that fusion protein resistant to ALK inhibition [133]. It also corresponds to the amino acid residues T315 in BCR-ABL and T790 in EGFR which are involved in acquired resistance [133]. The L1196M mutation is hypothesized to induce steric interference of crizotinib binding similar to those in BCR-ABL and EGFR [134, 135]. An *in vitro* model of EML4-ALK with the L1196M mutation showed that the cells were still dependent on ALK mediated signaling for tumor maintenance but are resistant to ALK inhibition [132, 136]. Several other drugs have shown activity in

inhibiting mutations induced by crizotinib and are in various stages of pre-clinical and clinical testing [137].

ALK Copy Number Gain and Emergence of ALK - Fusion Negative Tumors

In addition to the secondary gate keeper mutations, it appears that an increase in *ALK* gene copy numbers can occur after crizotinib treatment resulting in resistance. Several reports have identified 4-5 fold amplification of *EML4-ALK* in NSCLC after crizotinib treatment [136, 138, 139]. These patients with the copy number increases tend to progress on ALK inhibitor therapy and should be considered for different therapies. On the other hand, it has also been reported that some patients who previously tested positive for *EML4-ALK* rearrangements before therapy no longer show evidence of the fusion gene when tested with FISH or RT-PCR analysis [139]. Interestingly, some of these tumors now harbor exon 20/21 EGFR or KRAS G12C/G12V mutations that were absent before ALK inhibitor treatment [138, 139]. These tumors appear to have switched oncogenic drivers as a mechanism of ALK inhibitor resistance and the patients may benefit from EGFR treatment. Conversely, some of these fusion negative tumors do not show any secondary drivers and therefore the mechanism of resistance remains unknown. Understanding the genetic changes associated with crizotinib resistance in these patients will be important in determining what patients would be of benefit.

Heat Shock Protein (Hsp90) Targeting in EMK4-ALK Positive Tumors

A potential mechanism of overcoming EML4-ALK secondary mutation resistance involves the use of inhibitors to the protein folding chaperone, heat shock protein (Hsp90). Hsp90 is abundantly expressed and some of its known substrates are steroid hormone receptors and kinases involved in signal transduction [140]. Hsp90 is considered a “cancer chaperone” and an emerging candidate in cancer therapeutics as some of its well known clients include HER2, mutant KIT, EGFR and BCR-ABL [141]. It is required for the stability of these aberrantly activated/expressed proteins. Hsp90 inhibitors have reported activity against both the native and mutant fusion proteins in NSCLC [132, 142]. The inhibitors rapidly degrade EML4-ALK resulting in downstream signaling pathway inhibition, induction of cell growth arrest and apoptosis [132, 143]. In the clinic, Hsp90 inhibition has produced clinical responses in ALK positive patients who have failed crizotinib therapy [143, 144].

Role of Multidrug Resistance Proteins in NSCLC

Multidrug resistance (MDR) is a well characterized broad pattern of cross resistance to various structurally unrelated drugs after exposure to a single drug observed in *in vitro* culture models and in the clinic. The MDR phenotype can arise as a result of cellular adaptations including reduced drug uptake, increased drug efflux, alterations in intracellular drug distribution and inadequate induction of apoptosis [40, 145]. To date, the mechanism most associated with efflux of cytotoxic compounds involves membrane transport proteins. Membrane proteins belonging to the ATP binding cassette (ABC) transporter super-family have been found to actively expel a wide array of cytotoxic compounds in a process dependent on ATP hydrolysis [146]. The multidrug transporter MDR1 or P-glycoprotein (P-gp/ABCB1) is the most well characterized of these transmembrane efflux pumps and has been linked to drug resistance in mammalian cell lines and human tumors [147]. Modulation of the transport properties of P-gp has been a popular investigation point with various strategies of overcoming P-gp-mediated resistance proposed, but for the most part, these efforts have been futile [148, 149]. Other ABC transporters such as Multidrug Resistance Protein 1 (MRP1/ABCC1) [150], and Breast Cancer Related Protein (BCRP/ABCG2) [151] have also been investigated for potential roles in chemoresistance due to their drug efflux activities. These transporters have broad substrate specificity which can explain their roles in cross resistance, but these characteristics also makes them ideal candidates for investigation in both intrinsic and acquired resistance in NSCLC. In this section, we examine the roles of ABCB1, ABCC1, ABCG2 and ABCC10 in NSCLC chemoresistance.

ABCB1/MDR1/Pgp

ATP binding cassette, sub family B, member 1 (ABCB1) also known as multidrug transporter (MDR1) or P-glycoprotein (P-gp) was the first human ABC transporter characterized and implicated in the development of multidrug resistance [152]. It is expressed at low levels in most tissues but it is highly expressed in organs involved with barrier functions or excretion such as the liver, blood-brain barrier, placenta and the intestines [40, 153]. ABCB1 transports hydrophobic substrates, lipids, steroids, antibiotics, antihistamines, anticancer drugs such anthracyclines, *vinka* alkaloids and taxanes [153, 154]. *ABCB1* overexpression has been identified in many

forms of cancer and has been conclusively linked to poor treatment outcomes in breast cancer [155], and acute myelogenous leukemia (AML) [156].

In the normal lung, ABCB1 expression has been detected on the apical side of epithelial cells of the trachea and major bronchi [157, 158]. In lung cancer, evidence showing a significant role of *ABCB1* overexpression in chemoresistance has been contradictory. These conflicting results obtained during mRNA analysis by reverse transcription-polymerase chain reaction and protein analysis by immunohistochemistry may be due to several factors [159]. Contamination of tumor samples with normal tissue, poor sensitivity, specificity and quantitation difficulties are culprits for these contradictory findings. To address these issues, uniform analytical criteria need to be established.

Lai *et al*., reported low messenger RNA (mRNA) expression in analyses of 67 cell lines and 24 tumor samples. Relative higher expression was only observed in tumors with neuroendocrine markers. In the study, no correlation between *ABCB1* expression and *in vitro* cell line chemosensitivity, prior therapy status of the patients or clinical therapy outcomes was reported [160]. Immunohistochemical analysis of ABCB1 in tumor samples showed low heterogeneous expression only in 3-15% of tumor samples with no correlation to prognosis [161, 162]. In a disparate report, Beer *et al*., surprisingly detected expression in 34.6% of chemotherapy or radiotherapy naïve adenocarcinomas by immunohistochemistry [163]. They also reported more prominent expression on the invasion front of the tumors suggesting a possible of P-gp in enhancing the invasive potential of tumor cells.

Using patient derived xenografts not selected for resistance, Merk *et al*. examined the role of the intrinsic expression of various transporters including *ABCB1* in resistance to drugs such as etoposide, carboplatin and paclitaxel or targeted agents such as erlotinib [164]. In this report, there was no correlation between the expression of *ABCB1* mRNA and chemo-sensitivity. These findings contradict those by Choiu *et al*., reporting that indeed, there is a correlation between ABCB1 expression and paclitaxel response in patients [165]. Other studies have also reported that high expression of ABCB1 at protein and gene levels results in decreased drug sensitivity *in vitro, in vivo* [166] and in the clinic [167].The contradictory nature of these studies examining ABCB1 expression and roles in resistance in lung cancer suggest that further investigation is required to substantiate the importance of this transporter in this disease.

ABCB1 expression in lung cancer may have roles in chemoresistance through interactions with TKIs. It has been reported that ABCB1 can induce resistance to BCR-ABL and EGFR TKIs by reducing drug uptake [168, 169]. Some TKIs have been shown to activate ABCB1's ATPase activity and are transport substrates at low concentrations [170]. This is supported by observations demonstrating that knockdown of *ABCB1* by small interference RNA (siRNA) increases the intracellular concentrations of TKIs such as imatinib and erlotinib [171, 172]. Other TKIs such as nilotinib do not appear to be strong ABCB1 substrates as siRNA knockdown does not result in increased accumulation suggesting that there is selectivity in TKI transport by these pumps [173, 174]. Moreover, it has also been reported that some TKIs can upregulate ABC transporters including ABCB1 [175]. Harmsen *et al*., have described the upregulation of ABCB1 by gefitinib, erlotinib, sorafenib and nilotinib after 48 hour treatment with clinically relevant concentrations [175]. ABCB1 induction in this report was mediated by the nuclear receptor pregnane X. This upregulation affects the accumulation of ABCB1 substrates *in vitro* suggesting that this may be a factor in drug resistance. Conversely, other studies have shown that TKI's such as gefitinib and erlotinib inhibit the transport functions of ABCB1 at clinically achievable levels thus reversing resistance to cytotoxic drugs *in vitro* by increased accumulation and decreased efflux [176, 177]. It therefore appears that for therapeutic benefit in the clinic, factors such as transporter expression and usage of non-toxic but clinically relevant doses of the TKI to inhibit the efflux function of the transporter would need to be considered before regimen administration.

ABCC1/MRP1

ATP binding cassette, sub family C, member 1 (ABCC1) also known as Multidrug resistance protein 1 (MRP1) is an efflux pump originally discovered in doxorubicin resistant lung carcinoma cells displaying a multi-drug resistant phenotype without ABCB1 expression [150]. ABCC1 is expressed ubiquitously with particularly higher expression detected at the blood-brain barrier, intestines, choroid plexus and oral mucosa [178, 179]. ABCC1 expression in the lung is higher than in any other solid organs and thus it may have protective roles against air pollution and inhaled toxins [180, 181]. ABCC1 transports physiological substrates including leukotriene C_4, glutathione

conjugates, bile acids, folic acid but also confers resistance to drugs such doxorubicin, methotrexate, etoposide and vincristine [182, 183].

ABCC1 expression in lung tumors has been reported by various groups utilizing different techniques. Using Northern blotting, Ota *et al*. identified *ABCC1* expression in 31.6% of NSCLC, predominantly in squamous cell carcinoma and correlated with poor response to etoposide and vindesine [184]. Sugawara *et al*. demonstrated by immunohistochemistry that *ABCC1* was more abundantly expressed in adenocarcinomas than squamous and large cell carcinomas [185]. Increased *ABCC1* overexpression in lung cancer cells has been linked to increased copy number of chromosome 16 on which the *MRP1* gene is located [186]. A report by Doubre *et al*., showed 3-fold higher *ABCC1* expression level in NSCLC whose aneuploid cells showed a gain of chromosome 16 in diploid normal and carcinomatous cells [186]. No correlation however has been established between *ABCC1* expression and clinico-pathological parameters such as sex, age, smoking history, histology and tumor staging [187]. In the clinic, a strong correlation has been reported between high expression and negative response to cisplatin doublet therapy with vinorelbine, gemcitabine and paclitaxel [187, 188]. In addition, ABCC1 expression can signify overall poor prognoses in patients, a decrease in progression free survival and overall survival [184, 188]. It thus appears that ABCC1 may have strong implications for management of NSCLC and not surprisingly design of ABCC1 specific inhibitors and modulation of ABCC1 as a therapeutic measure is under active investigation [189, 190].

ABCC10/MRP7

ATP binding cassette, sub family C, member 10 (ABCC10) also known as Multidrug resistance protein 1 (MRP7) is widely expressed in most tissues at low levels [191-193]. As an efflux pump, ABCC10 extrudes a wide range of anticancer drug substrates. It transports and confers resistance to taxanes, *vinka* alkaloids, anthracyclines and epothilone B [194, 195]. In a knockout mouse model, Abcc10 has been confirmed to be a major efflux pump for paclitaxel *in vivo* [196]. Upon exposure to sublethal doses of paclitaxel, these *Abcc10*$^{-/-}$ mice exhibited significant damage to lymphoid and hematopoietic tissue.

Up-regulated expression of *ABCC10* has been detected in tumors including pancreatic adenocarcinoma [197], colorectal cancer [198] and NSCLC [199]. In colorectal cancer, an inverse correlation between *ABCC10* expression and clinical tumor grading has been reported [198]. In this disease, tumor aggressiveness is associated with a decrease in transcript levels [198]. In a study by Wang *et al*., characterizing expression in 155 matched normal and NSCLC surgical specimens, ABCC10 was localized to the membrane and cytoplasm in normal lung tissue, lung adenocarcinoma and squamous cell carcinoma [199]. Significantly, normal tissue showed little to no ABCC10 expression, squamous cell carcinoma moderate expression while adenocarcinomas showed the highest expression. Additionally, differences were noted in the pathological staging of the adenocarcinomas with TNM stage III tumors showing significant more expression than stage I and II tumors. This study by Wang *et al*. is important as it offers the first characterization of ABCC10 expression in a large panel of untreated NSCLC tumor specimens.

In addition to this study, several *in vitro* studies support a role of ABCC10 in lung cancer. Oguri *et al*. have reported that *ABCC10* may be a predictive biomarker for paclitaxel resistance in lung cancer cell lines [200]. Analysis of the paclitaxel-selected and resistant subline PC-6/TAX1-1, showed higher expression of *ABCB1* and *ABCC10* when compared to parental PC-6 cells. An inverse correlation was established between the expression of *ABCC10* and sensitivity to paclitaxel *in vitro* in PC-6/TAX1-1 cells and in an additional panel of 17 NSCSL cell lines, 13 of which did not express *ABCB1*. Pretreatment of the NSCLC cells with a non-specific ABCC subfamily inhibitor, sulfinpyrazone led to enhanced cytotoxicity of paclitaxel and increased intracellular accumulation of the drug. In a separate study, *ABCC10* has been suggested to be a marker for vinorelbine resistance in NSCLC [201]. Up-regulated expression of *ABCC10* was observed in vinorelbine selected NSCLC cell lines. This upregulation was associated with decreased sensitivity to the drug and was reversed by *ABCC10* siRNA. Given that most of the *in vitro* studies reported on ABCC10 in lung cancer are from drug selected cell lines, further investigation on non-drug selected backgrounds are required.

ABCG2/BCRP

ATP binding cassette, sub family G, member 2 (ABCG2) also known as Breast cancer related protein is an ABC half transporter originally identified in a MCF7 human breast cancer subline resistant to anthracyclines [151]. In this study, *ABCG2* expression

resulted in reduced accumulation of daunorubicin, and increased resistance to anthracyclines and mitoxantrone [151]. ABCG2 expression also mediates resistance to topoisomerase 1 inhibitors such as SN-38, irinotecan and topotecan [202, 203]. It has however not been shown to confer resistance to paclitaxel, cisplatin or vinka alkaloids [151, 204]. *ABCG2* expression has been identified in the epithelium of small intestines and colon, liver, breast ducts and lobes, in vein and capillary epithelium, blood-brain barrier, blood-testis barrier suggesting roles in tissue protection and regulation of drug uptake from the gut [205, 206].

In the normal lung, ABCG2 protein expression is low and lesser than ABCB1 and ABCC1 with the expression localized to the epithelial cell layer, seromucinous gland and small capillaries [203, 207]. In tumors, strong ABCG2 expression has been detected in a majority of malignancies with immunoreactivity observed in over 10% of the cells localized in both the membrane and cytoplasm [208]. ABCG2 expression seems to have the highest prognostic value in leukemia and is commonly linked to therapy failure [206]. In NSCLC, ABCG2 expression appears to show the greatest clinical correlation with low responses to platinum based agents [209]. *ABCG2* expression and correlation with sensitivity to other standard cytotoxic therapies in NSCLC is not compelling. In a study using patient derived xenografts, expression was not a significant player in the response to paclitaxel, carboplatin, gemcitabine and erlotinib and mRNA expression only correlated with etoposide sensitivity [164].

ABCG2 expression in NSCLC shows some association with acquired resistance to TKIs and similar to observations in ABCB1, low concentrations of TKIs stimulate ABCG2 ATP hydrolysis, but inhibit transport at higher concentrations [210]. In NSCLC, it has been reported that ABCG2 expression may be a factor in gefitinib resistance, but it is important to note that gefitinib is both a transport substrate and inhibitor of ABCG2 [211]. In a case reported by Usuda *et al*., despite initial response, the disease progressed while on gefitinib and while no secondary EGFR mutations were found, ABCG2 expression was detected in the recurrent tumor and was suggested to be the cause of resistance [212]. Studies to elucidate the mechanism of ABCG2 expression and correlation with resistance to EGFR inhibitors found elevated levels of wild type EGFR in the nucleus [213]. This increased expression was associated with increased phosphorylation of AKT at Ser-229. In the nucleus, EGFR targets the *ABCG2* promoter and enhances its expression. These findings illustrate the complex genomic interactions involved in resistance mechanisms. The poor clinical outcome reported indicates that ABCG2 may be a negative predictive factor in patients with wild-type EGFR and that it could be a potential target to increase sensitivity to these inhibitors. On the contrary, there is a positive link between ABCG2 and tyrosine kinase inhibitors as erlotinib, gefitinib and AG1478 have been shown to antagonize ABCG2 transport functions [177, 210, 214]. Similar to ABCB1, how to harness the ABCG2 inhibitory effects of TKIs for resistance reversal requires further investigation.

Active research continues to identify the roles of ABC transporters in NSCLC and lung biology but hurdles exist. Most of the information currently available on the expression of ABC transporters in normal and cancerous lung has been obtained from archival tissue and cell line studies due to lack of lung-tissue specific animal models. Lung-specific engineered mouse models of ABC transporters would be beneficial in answering queries about roles in development given the complex lung architecture, the substrates of these transporters in lung and bronchial tissues as well as roles in lung cancer biology and chemoresistance. One of the other hurdles faced in the effort to develop inhibitors for the ATP transporters has been lack of specificity. For instance, ABCB1 inhibitors such as cepharanthine and tariquidar also reverse ABCC10 mediated resistance [215, 216]. Additionally, tyrosine kinase inhibitors such as lapatinib, nilotinib and erlotinib are reversal agents for ABCC10 resistance but they also inhibit the activity of ABCB1 and ABCG2 *in vitro* [217, 218]. However, questions about this off-target activity of TKIs against ABC transporters and how it relates to tyrosine kinase receptors exist and need to be resolved. Furthermore given the ABC transporter targeting activity of these TKIs, they could serve as backbones for the design of novel ABC transporter inhibitors. With the emerging roles of ABC transporters in NSCLC, it is to be expected that in the future, understanding how to successfully target the efflux functions of these proteins may provide another strategy to increase the survival and promote positive treatment outcomes of NSCLC patients.

CONCLUSION

In this review, we have covered some of the causes of intrinsic and acquired resistance in NSCLC. The available genetic information correlating to positive or

negative clinical parameters expands the field of pharmacogenetic driven therapies. Given that this disease is highly refractory to therapy, understanding how to tailor systemic or targeted therapy to patients sharing a common genetic marker can significantly improve benefit. Pharmacogenetic testing is already incorporated in some clinical settings during clinical trials or during treatment with already validated clinical markers such as EGFR, KRAS and ALK. The cost of biomarker testing and the difficulty in obtaining enough metastatic NSCLC tissue are just two of the limitations for widespread pharmacogenetic testing in NSCLC. In the future, the use of readily accessible specimens such as blood for biomarker testing in NSCLC would greatly improve therapeutic prediction. Furthermore, the creation of a complex biomarker array system based on a combination of several predictive markers would be beneficial given that combination therapies are more commonly used than monotherapies.

Additional research is required to clear the discrepancies and contradictory studies published involving the role of ABC transporters in NSCLC. ABC transporters appear beneficial not just as candidates for enhancement of intracellular drug retention, but they may be an emerging class of predictive and prognostic markers. Lack of effective clinical application of inhibitors of ABC transporters remains a big challenge despite efforts put into development of inhibitors. Given the dismal survival rate of this disease, all aspects that affect tumor response to chemotherapeutic agents need to be investigated to see how they can be harnessed to improve clinical responses.

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