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PII: S0163-7258(19)30190-1

DOI: https://doi.org/10.1016/j.pharmthera.2019.107438

Reference: JPT 107438

To appear in: *Pharmacology and Therapeutics*

Please cite this article as: W.-j. Liu, Y. Du, R. Wen, et al., Drug resistance to targeted therapeutic strategies in non-small cell lung cancer, *Pharmacology and Therapeutics*(2019), https://doi.org/10.1016/j.pharmthera.2019.107438

P&T #23609

Drug resistance to targeted therapeutic strategies in non-small cell lung cancer

Wen-juan Liu[1], Yue Du[2], Ru Wen[3], Ming Yang[1,**] aaryoung@yeah.net and Jian Xu[4,5,*] jian.xu@nih.gov

[1]Shandong Provincial Key Laboratory of Radiation Oncology, Shandong Cancer Hospital and Institute, Shandong First Medical University and Shandong Academy of Medical Sciences, China.

[2]Department of Pharmacy, the First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China

[3]Department of Medicine, Stanford University School of Medicine, California, USA

[4]Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, Bethesda, MD, USA

[5]Lead Contact

*Correspondence to: Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, Bethesda, MD, USA.

**Correspondence to: Shandong Provincial Key Laboratory of Radiation Oncology, Shandong Cancer Hospital and Institute, Shandong First Medical University and Shandong Academy of Medical Sciences, China.

Abstract

Rapidly developing molecular biology techniques have been employed to identify cancer driver genes in specimens from patients with non-small cell lung cancer (NSCLC). Inhibitors and antibodies that specifically target driver gene-mediated signaling pathways to suppress tumor growth and progression are expected to extend the survival time and further improve the quality of life of patients. However, the health of patients with advanced and metastatic NSCLC presents significant challenges due to treatment resistance, mediated by cancer driver gene alteration, epigenetic alteration, and tumor heterogeneity. In this review, we discuss two different resistance mechanisms in NSCLC targeted therapies, namely changes in the targeted oncogenes (on-target resistance) and changes in other related signaling pathways (off-target resistance) in tumor cells. We highlight the conventional mechanisms of drug resistance elicited by the complex heterogeneous microenvironment of NSCLC during targeted therapy, including mutations in epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), the receptor tyrosine kinase ROS proto-oncogene 1 (ROS1), and the serine/threonine-protein kinase BRAF (v-Raf murine sarcoma viral oncogene homolog B). We also discuss the mechanism of action of less common oncoproteins, as in-depth understanding of these molecular mechanisms is important for optimizing treatment strategies.

Keywords: Non-small cell lung cancer; Drug resistance; Gene alteration; Tumor microenvironment; Therapeutic strategy

Abbreviations

NSCLC Non-small cell lung cancer

EGFR Epidermal growth factor receptor

ALK Anaplastic lymphoma kinase

ROS1 Receptor tyrosine kinase ROS proto-oncogene 1

BRAF Serine/threonine-protein kinase BRAF

MET Mesenchymal-epithelial transition

HER2 Human epidermal growth factor receptor 2

TKI Tyrosine kinase inhibitor

mTOR Mammalian target of rapamycin

PFS Progression-free survival

OS Overall survival

ORR Objective response rate

IGF Insulin-like growth factor

DoR Duration of response

EMT Epithelial-mesenchymal transition

HDAC Histone deacetylase

HAT Histone acetyltransferase

KMT Histone lysine methyltransferases

KDM Histone lysine demethylases

LncRNA Long non-coding RNA

TME Tumor microenvironment

ECM Extracellular matrix

CAF Cancer-associated fibroblast

MMPs Matrix metalloproteinases

VEGF Vascular endothelial growth factor

ICI Immune checkpoint inhibitor

PD-L1 Programmed cell death-ligand 1

PD-1 Programmed cell death protein 1

TAM Tumor-associated macrophage.

1. Introduction

In 2018, approximately 18.1 million new cases of cancer and 9.6 million cancer deaths were reported. Lung cancer ranks first in terms of morbidity and mortality worldwide (Bray et al., 2018). The number of lung cancer-related deaths in 2018 was estimated to be 1.8 million, which accounts for nearly one-fifth of all cancer-related deaths (Bray et al., 2018). Non-small cell lung cancer (NSCLC) is the most predominant pathological subtype of lung cancer, accounting for approximately 85% in all cases (Rotow and Bivona, 2017; Thomas et al., 2015). NSCLC includes squamous cell carcinoma, adenocarcinoma, and large cell carcinoma; of these, adenocarcinomas grow slowly and have relatively low diffusion and metastatic activity. Currently, two-thirds of clinical specimens from patients with NSCLC harbor gene alterations, and targeted therapeutic drugs are available for half of these alterations (Rotow and Bivona, 2017). Compared with therapies based on chemotherapeutic drugs alone, targeted drugs significantly improve survival time and the quality of life of patients (Maman and Witz, 2018; Park and Han, 2019). National Institute for Health and Care Excellence (NICE) recommended docetaxel plus platinum or platinum-doublet for the treatment of patients with locally advanced or metastatic NSCLC. Studies have also shown that although the benefits of adjuvant chemotherapy in patients with early-stage NSCLC are limited, platinum-based chemotherapy is still recommended in patients with stage II, stage III, and stage IB with tumor size over 4 cm, the 5-year survival rate is only about 5% (Nagasaka and Gadgeel, 2018). Therapeutic resistance limits the clinical use of chemotherapy drugs in patients with advanced NSCLC. For example, the main mechanisms of resistance of paclitaxel or platinum-based chemotherapy drugs include insufficient drug level to target DNA, lack of DNA repair pathway, and the SRC-activated ERK pathway (Pilkington et al., 2015). Recent

studies have focused on improving cisplatin delivery to reduce toxicity to patients (Fennell et al., 2016).

Over the past decade, the heterogeneity of advanced NSCLC has become more complex and many genetic drivers have been identified as pivotal oncogenic factors. NSCLC genotypes with significant responses to treatment include those with epidermal growth factor receptor (EGFR) mutations, anaplastic lymphoma kinase (ALK) mutations, ROS proto-oncogene 1 (ROS1) rearrangement, mesenchymal-epithelial transition (MET) factor amplification, v-Raf murine sarcoma viral oncogene homolog B (BRAF*)* mutations, human epidermal growth factor receptor 2 (HER2) mutations, and RET rearrangement (Molina et al., 2008; Rotow and Bivona, 2017; Schrank et al., 2018; Westover et al., 2018). Activated EGFR mutations occur in only 10-20% of patients with NSCLC in North America and Europe (Kobayashi et al., 2005; Westover et al., 2018), increasing to 60% among Asian patients (Nahar et al., 2018; Westover et al., 2018). Compared with standard chemotherapy, treatment of EGFR-mutant lung cancers with tyrosine kinase inhibitors (TKIs) specifically targeting EGFR significantly inhibits tumor growth, prolongs progression-free survival (PFS), and improves quality of life (Schrank et al., 2018). Patients with ALK gene rearrangement respond well to ALK-directed TKI therapy, which markedly improves PFS compared with chemotherapy (Soda et al., 2007). Administration of targeted inhibitors improves the prognosis of patients with NSCLC (Deeks, 2016; Rossi, 2016); however, patient response to these drugs is generally short-term, with the majority developing drug resistance after one year.

Resistance to targeted drug therapy may be primary (intrinsic) resistance or acquired resistance. The mechanisms of resistance can also be classified as on-target and off-target (Rotow and

Bivona, 2017). On-target resistance occurs when the primary molecular target of the drug is mutated with poor or no response to treatment, while off-target resistance develops by activating signaling pathways parallel to the target of interest or bypass signaling pathways downstream of the target of interest. Recent studies have shown that rapid, multi-level epigenetic alterations of gene expression profile in tumors represent one of the most important reasons of drug resistance (Park and Han, 2019). In many circumstances, the gene expression pattern constantly evolves under selection during treatment, resulting in the development of acquired resistance. Epigenetic alterations typically help tumor cells to escape host immune surveillance and contribute to resistance to therapeutic drugs. A number of studies have started focusing on drug resistance caused by epigenetic alterations, developing different drugs to target DNA methylation or histone modifications (Park and Han, 2019; Schiffmann et al., 2016). The growth and progression of tumors also depend on external environment. The interaction between tumor cells and their surrounding components is another important reason of drug resistance. Finally, changes in drug absorption or exposure are considered to be approaches of drug resistance (Sun et al., 2018). In this review, we discuss the current understanding of resistance mechanisms to therapies that target NSCLC oncogenes. We highlight drug resistance elicited by different intracellular changes and changes in the external components of tumor cells. An in-depth understanding of the mechanisms of drug resistance provides a broader perspective for developing more effective small-molecule inhibitors or antibodies, and also provides a theoretical basis for designing combined regimens of chemotherapeutic and targeted drugs. These optimized inhibitors, antibodies, and combinations have great potential to direct targets of interest as well as drug-resistance mutations.

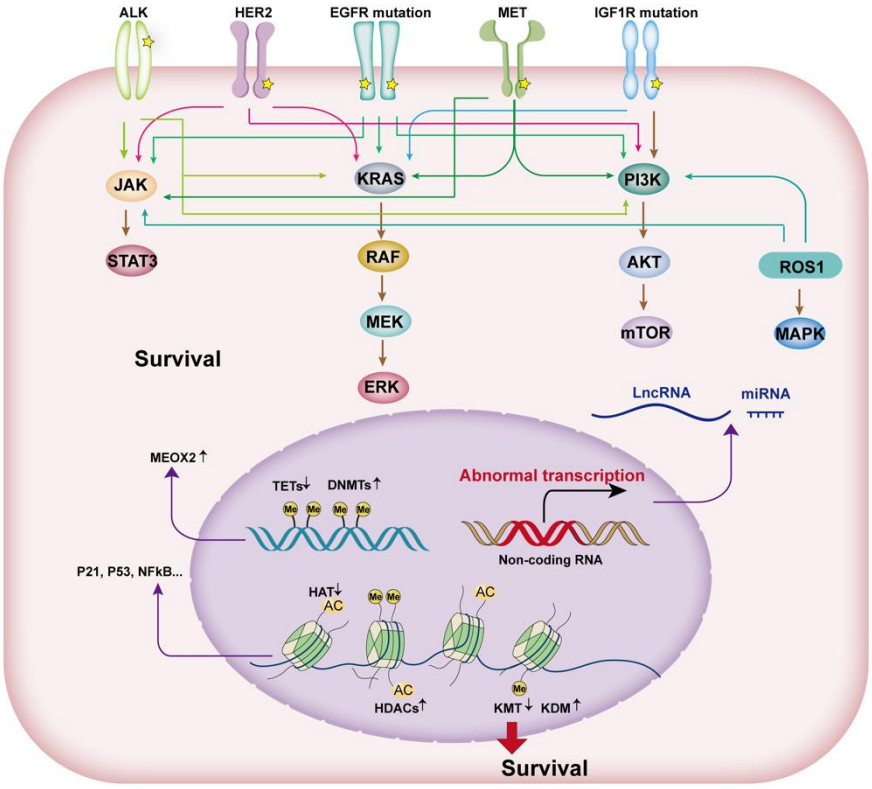

Figure 1 Gene alterations associated with drug resistance in NSCLC

2. Gene alterations associated with drug resistance

Changes in the surface proteins of tumor cells are closely correlated with the emergence of drug resistance (Westover et al., 2018). In addition to the widely studied EGFR and ALK, changes in many other oncogenic driver factors are also common in NSCLC, such as ROS1 rearrangements, BRAF mutations, RET rearrangements, and mutual mutations in multiple oncogenic driver factors (Figure 1). Moreover, changes in downstream signaling pathways may bypass inhibitor-mediated blockage of oncogenes, leading to drug resistance.

2.1 EGFR-TKIs

Abnormalities of EGFR, a tyrosine kinase receptor, generally activate the phosphoinositide

3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) and RAS/RAF/MEK signaling pathways. These induce pathological cell proliferation and anti-apoptotic activity, promoting tumor growth and progression (Cheng and Chen, 2014). Abnormalities in EGFR primarily include gene mutation, gene amplification, and protein overexpression (Hofman and Hofman, 2019; Westover et al., 2018). A relatively high EGFR mutation frequency (30-60%) is observed in patients with NSCLC. Approximately 90% of EGFR mutations occur in exon 19 (exon 19 deletion mutations) and exon 21 (L858R) (Camidge et al., 2014; Robichaux et al., 2018). To date, the US Food and Drug Administration (FDA) has approved the following four EGFR TKIs for clinical treatment, the first-generation TKIs erlotinib (Higgins et al., 2004) and gefitinib (Santoro et al., 2004), the second-generation TKI afatinib (Hirsh, 2011), and the recently approved osimertinib, which is a third-generation and mutant EGFR-specific TKI (Soria et al., 2018). The objective response rate (ORR) of first-line treatment is 50-80% (Soria et al., 2018). From the molecular and clinical perspective, drug resistance during treatment with EGFR inhibitors can be divided into primary resistance and acquired resistance.

Patients with EGFR mutations with primary resistance experience poor treatment efficacy from the time of treatment initiation (Zupa et al., 2012). For instance, exon 20 insertions or duplications, accounting for 4% of all EGFR mutations, are mostly insensitive to the existing EGFR tyrosine kinase inhibitors (TKIs) (Zupa et al., 2012). Analysis of patients with EGFR exon 20 insertion mutations shows that the ORRs to first-line treatment with erlotinib, gefitinib, or afatinib are within 3-8% (Sutiman et al., 2017). Through the clinical database, Robichaux et al. (2018) analyzed the response of NSCLC patients with EGFR exon 20 insertion mutations to TKIs, and found that among 280 patients with EGFR mutations, classical mutations were detected in 129 patients

(exon 19 deletion, p.L858R mutation, and p.L861Q mutation), while EGFR exon 20 insertion mutations were only detected in 9 patients. Patients with NSCLC were administered erlotinib, gefitinib, or afatinib monotherapy, and those with classical EGFR mutations had a median PFS of 14 months, while patients with EGFR exon 20 insertion mutations had a median PFS of only 2 months (Robichaux et al., 2018). These clinical data indicated that EGFR TKIs have very limited therapeutic effects on NSCLCs driven by EGFR exon 20 insertion mutations. Primary resistance to EGFR TKIs may also be induced by a combination of sensitizing EGFR mutations and secondary genetic mutations. Finally, genetic alterations that occur outside the target kinases may also reduce sensitivity to EGFR TKIs and cause progression of primary resistance. For example, MET amplification, BIM expression reduction, and nuclear factor kappa B (NF-kB) signaling pathway activation can reduce EGFR TKI treatment efficacy (Cappuzzo et al., 2009; Lee et al., 2013; Sos et al., 2009). Acquired resistance is a common mode of resistance in NSCLC, and the condition of patients with NSCLC with EGFR mutations continues to deteriorate even after treatment, developing resistance to TKIs (Robichaux et al., 2018). The EGFR exon 19 deletion and L858R point mutations in exon 21 are associated with the anti-tumor activity of EGFR-TKIs (Yang et al., 2011; Yang et al., 2015). A secondary mutation in EGFR exon 20 results in a threonine mutation at position 790 to methionine (T790M), which is a most common molecular mechanism of resistance (Kobayashi et al., 2005; Pao et al., 2005). In a 2005 study, patients with lung adenocarcinoma experienced disease progression after gefitinib treatment (Pao et al., 2005). The occurrence of the T790M mutation was observed in the EGFR kinase domain causing secondary drug resistance (Pao et al., 2005). EGFR-mutant tumors are usually sensitive to TKIs, because the mutation reduces the affinity of the receptor to ATP (Pao et al., 2005);

however, the T790M mutation alters the affinity of EGFR tyrosine kinase to ATP and restores ATP affinity to wild type (WT) level (Yun et al., 2008). As a result, the mutated EGFR uses ATP as a substrate instead of TKIs. A further example is EGFR amplification, which occurs in approximately 10% of patients with acquired resistance to EGFR TKIs (Ercan et al., 2010). Other non-T790M drug-resistant mutations of EGFR are clinically rare, accounting for 1-2% of all EGFR mutations (Sequist et al., 2011). Such mutations include T854A (Bean et al., 2008), D761Y (Balak et al., 2006), L797S, and L747S (Yamaguchi et al., 2014), which have also been described in patients with secondary drug resistance. EGFR mutations also include EGFR-G719X (X represents any other amino acid) (Katono et al., 2013), and EGFR-L861X (Rotow and Bivona, 2017). However, the response rate of these patients to EGFR TKI treatment are not known. Osimertinib is a third-generation EGFR TKI with high selectivity that remains the preferred second-line choice for patients with T790-mediated resistance to first or second-generation EGFR TKIs (Soria et al., 2018; Jeong and Um, 2019).

The third generation TKI-resistant EGFR secondary mutations include C797S or E709K, L692V and L798I (Niederst et al., 2018). The amplification of MET and insulin-like growth factor 1 receptor (IGF1R), as well as the mutation or amplification of genes involved in signal transduction, such as BRAF, are also regarded as the mechanisms of third generation TKI resistance (Le et al., 2018). Osimertinib specifically inhibits mutated-EGFR through irreversible and covalent binding to C797 in the TK domain (Niederst et al., 2018). A Phase 3 trial comparing osimertinib with chemotherapy in patients who developed acquired resistance caused by T790M after first-line EGFR-TKI treatment (AURA3 trial), found that osimertinib was associated with a greater progression-free survival (PFS) (10.1 months versus 4.4 months).

EGFR bypass pathway activation also leads to secondary drug resistance in 20% of patients with NSCLC, activating the same EGFR downstream effectors which are crucial for tumor cells growth through different pathways (Table 1). EGFR is a membrane protein that affects cellular functions by activating downstream signaling pathways, however, abnormalities in other membrane proteins that activate signaling events downstream of EGFR may also cause resistance to EGFR TKIs. For example, MET amplification activates the PI3K/AKT/mTOR signaling pathway (Ito et al., 2019), and overexpression of hepatocyte growth factor (HGF, MET ligand) is also associated with the development of EGFR TKI resistance. Another important membrane receptor HER2, functions in a downstream signaling pathway similar to the EGFR pathway (Peifer et al., 2012). Its abnormal amplification also results in secondary drug resistance. In addition, mutations in certain proteins in the EGFR downstream signaling pathway also mediate EGFR TKI resistance. For example, mutated PIK3CA activates AKT, which was detected in a small number of patients with secondary drug resistance (Heavey et al., 2014). Studies have also shown that EGFR-driven lung cancers harboring other mutations (EGFR plus BRAF mutations, EGFR plus p53 mutations, EGFR plus MET mutations, or EGFR plus KRAS mutations) have a more rapid progression (Rotow and Bivona, 2017). The emergence of BRAF mutations may also represent the mechanism of acquired resistance to EGFR inhibitors in EGFR-mutant NSCLC. Ohashi et al. (2012) reported that among 195 patients, two cases with BRAF mutations developed acquired resistance to EGFR inhibitors. Of these, one patient developed BRAF-V600 and EGFR T790M mutations, while the second patient developed G469A BRAF mutation. The mesenchymal-epithelial transition factor (MET), a tyrosine kinase encoded by the proto-oncogene c-MET, is a HGF receptor (Reis et al., 2018). MET dysregulation usually occurs during the

development of resistance to EGFR-TKI therapy, representing a type of EGFR TKI resistance mechanism (Frampton et al., 2015). Approximately 5-26% of patients with NSCLC who are resistant to EGFR inhibitor therapy, carry MET amplification or/and MET overexpression, negatively affecting the outcome of EGFR-targeted therapy (Bahcall et al., 2018; Kim et al., 2018; Paik et al., 2015). Recent study indicated that MET is an attractive therapeutic target in NSCLC cancer, and MET amplification is also a good predictor of MET-TKI sensitivity (Reungwetwattana et al., 2017). Capmatinib (INC280, Novartis) is a specifically targeted and orally used MET inhibitor. In vitro studies have shown that Capmatinib inhibited MET phosphorylation and activated associated downstream effectors in MET-dependent tumor cells (Vansteenkiste et al., 2019). Capmatinib was also reported for highly potent tumor growth inhibition in MET-positive patients. In EGFR-mutant NSCLC, combination therapy with EGFR-TKI can achieve significant efficacy in drug-resistant patients with high MET amplification (Vansteenkiste et al., 2019). TATTON is an ongoing Phase Ib trial in which the third generation EGFR-TKI osimertinib is combined with MET inhibitor savolitinib in patients with NSCLC with EGFR mutations and MET amplification (NCT02143466). By February 2018, with disease (the first and second generation EGFR-TKIs) progression, osimertinib was administered in combination with savolitinib in 46 patients with NSCLC with EGFR mutations and MET amplifications. Treatment response (52% ORR) was observed in 24 cases, with a median duration of response (DoR) of 7.1 months. A further multicenter, open-label phase II trial of savolitinib for patients with NSCLC with MET Exon 14 mutation is currently in progress (NCT02897479).

2.2 ALK-TKIs

ALK is a transmembrane receptor tyrosine kinase that belongs to the insulin receptor superfamily. The majority of ALK-positive patients harbor the echinoderm microtubule-associated protein-like 4 (EML4)-ALK fusion gene rearrangement, accounting for 3-7% of all patients with NSCLC (Qian et al., 2017; Sharma et al., 2018). Among patients with the EML4-ALK fusion gene, approximately 33% show ALK mutations, 16% show ALK amplification, and 35% show the activation of bypass signaling pathways, such as EGFR, insulin-like growth factor (IGF), HGF, and neuregulin 1 (NRG1) (Hutchinson, 2016; Sharma et al., 2018; Spaans and Goss, 2014) (Table 1).

Crizotinib is a first-generation ALK inhibitor. Compared with chemotherapy alone, crizotinib leads to prolonged PFS and better ORR (Crystal and Shaw, 2011; Pearson and Kolesar, 2012). Acquired resistance to crizotinib usually emerges after 1 year of treatment. The known mechanisms of resistance include point mutation, fusion gene amplification, and bypass signaling via activation of other oncogenes, such as EGFR, MAP kinase-ERK kinase (MEK), extracellular signal-regulated kinase (ERK), and the SRC proto-oncogene (Solomon et al., 2014). Gainor (2016) detected the ALK E1210K mutation in patients treated with crizotinib. Further secondary mutations occurring after crizotinib treatment include L1196M, G1269A, G1202R, S1206Y, G1269A, L1152R, D1203N, I1171T, V1180L, and C1156Y (Friboulet et al., 2014; Toyokawa and Seto, 2015). Compared with crizotinib, the second-generation ALK inhibitors, ceritinib, alectinib, and brigatinib exhibit increased activity against ALK (Costa, 2014). In addition, they display improved CNS permeability and are able to target multiple secondary ALK mutations. A greater therapeutic effect can be obtained when patients are treated with second-generation ALK TKIs directly. Ceritinib is an oral ALK TKI with ATP-competitive activity that elicits a response in

approximately 40-50% of crizotinib-resistant patients, which results in a median PFS of 7-12 months (Deeks, 2016). In addition to ALK, ceritinib inhibits insulin-like growth factor 1 (IGF1), ROS1, and insulin receptors (Roskoski, 2017; Shaw and Engelman, 2014). Moreover, ceritinib inhibits a variety of crizotinib-resistant ALK mutations, including L1196M, G1269A, and S1206Y (Friboulet et al., 2014). However, C1156Y/T, I1151Tins, and L1152P/R mutations are associated with the emergence of ceritinib resistance (Rothschild, 2016).

Alectinib has a better efficacy against G1269A, L1196M, F1174L, and C1156Y mutations (Rossi, 2016). A meta-analysis of two single-arm, open phase II studies (NP28673, NCT01801111; NP28761, NCT01871805) revealed that alectinib exhibits potent therapeutic effects against crizotinib-resistant ALK-positive NSCLC as patients treated with alectinib had a median overall survival (OS) of 29.5 months (Morcos et al., 2018). However, alectinib treatment elicits the emergence of resistance mutations I1171T/N/S, V1180L, and G1202R (Gainor et al., 2016; Katayama et al., 2014). The activated bypass signaling pathways are mediated by HGF and MET (Isozaki et al., 2016). Epithelial-mesenchymal transition, characterized by the loss of E-cadherin and increased expression of vimentin, is a potential mechanism of alectinib resistance (Sequist et al., 2011). It appears that alectinib or ceritinib resistance cannot be overcome by other ALK inhibitors (Dong et al., 2016).

Brigatinib is a novel inhibitor targeting ALK, ROS1, and EGFR (Schaefer and Baik, 2016). An ongoing phase III clinical trial, ALTA-1L (NCT02737501), is comparing the efficacy and safety of brigatinib with crizotinib, when used as the first-line therapy in patients with ALK-positive metastatic NSCLC (Castellanos and Horn, 2016). Brigatinib inhibits crizotinib-resistant mutations, including ALK L1196M and EGFR T790M (Castellanos and Horn, 2016), however, the mechanism

of Brigatinib resistance remains unclear, as the drug is undergoing clinical trials.

In 2018, the new generation of ALK inhibitor Lorlatinib was approved by the FDA for ALK-positive NSCLC treatment. Lorlatinib can be used to treat all known ALK inhibitor-caused resistance mutations and is the preferred treatment in patients with alectinib resistance (Okada et al., 2019; Yoda et al., 2018). Recently, studies have also reported that Lorlatinib resistance may still be mediated by ALK-mutations (mutations on the same allele). Analysis of tumor biopsy from patients with Lorlatinib resistance showed that ALK-mutation was developed gradually in patients after ALK inhibitors treatment (Okada et al., 2019; Yoda et al., 2018).

2.3 Other targetable molecular mutations

2.3.1 Oncogenic ROS1 gene rearrangement

Based on the detection of specimens from patients with NSCLC, the probability of ROS1 gene rearrangement is 1-2% (Roskoski, 2017). The ROS1 kinase domain is capable of fusing with cluster of differentiation 74 (CD74), which leads to constitutive ROS1 kinase activity. Due to the structural homology between the ALK and ROS1 kinase domains, ALK TKIs can be used to cross-inhibit ROS1 kinase activity (Facchinetti et al., 2016). Crizotinib has been approved for the treatment of NSCLC with ROS1 rearrangement, and is associated with an ORR of 72% and a median PFS of 19.2 months (Shaw et al., 2014). Repotrectinib (TPX-0005) is one of next-generation ROS1 TKIs that has been used to treat patients with ROS1/TRK/ALK Solvent-Front Mutations (Drilon et al., 2018).

2.3.2 Oncogenic BRAF mutation

In addition to EGFR, ALK, and ROS1, BRAF represents a novel therapeutic target for the treatment of advanced NSCLC (Leonetti et al., 2018). Mutations in the BRAF gene act as an alternative carcinogenic driver in NSCLC. One study analyzing different BRAF mutation cases, revealed that V600 mutations are more likely to occur in light/non-smokers and are more common in female patients worldwide (Caparica et al., 2016). In contrast, non-V600E mutations occur almost exclusively in male patients. The frequency of BRAF mutations also shows a proportional correlation with age. In lung adenocarcinoma, the BRAF mutation rate is 3-8% (Davies et al., 2002; Leonetti et al., 2018; Naoki et al., 2002). These mutations cause permanent activation of MAPK pathways, promoting continuous tumor growth. Among the BRAF mutations, nearly 50% are BRAF-V600E, 35% are BRAF-G469A/V, and 7% are BRAF-D594G mutations (Tissot et al., 2016). The BRAF-V600E mutation is responsible for resistance to the third-generation EGFR TKI osimertinib by activating downstream MEK-ERK signaling (Ho et al., 2017). In 2016, the FDA approved BRAF-V600 inhibitors vemurafenib and dabrafenib in combination with the MEK inhibitor trametinib for the clinical treatment of BRAF-V600E-positive NSCLC (Chapman et al., 2011; Hauschild et al., 2012). In 2017, Leonetti et al. reported that among BRAF-V600-positive patients with lung cancer included in a multi-arm, phase II vemurafenib trial, vemurafenib was administered as a second-line treatment in 54 patients and as a first-line treatment in 8 patients (Leonetti et al., 2018). After a specified period, disease progression was diagnosed in 20 patients who were administered vemurafenib as a second-line treatment and 3 patients who were administered the drug as a first-line treatment. Patients administered vemurafenib as a first-line treatment achieved a median PFS of 12.9 months, while those administered vemurafenib as a second-line treatment showed a median PFS of 6 months (Leonetti et al., 2018).

In a phase II trial conducted by Planchard et al. (2016), 84 patients with BRAF-V600E NSCLC and previously treated patients were re-subjected to dabrafenib monotherapy. Among the 84 subjects, 26 (33%) achieved an objective response while 45 (58%) achieved disease control. The median PFS and the median DoR were 5.5 and 9.6 months, respectively. Among the 6 patients who received dabrafenib as a first-line treatment, 4 achieved an objective response. The remaining 2 subjects were followed-up for 29 months. The patients had a median OS of 12.7 months and the 1 and 2-year survival rates were 52% and 31%, respectively (Leonetti et al., 2018; Planchard et al., 2016). One year later, another phase II trial by Planchard et al. (2016) reported a median PFS of 10.9 months and a median DoR of 10.4 months among 36 patients treated with dabrafenib and trametinib as a first-line therapy; OS of the patients was 24.6 months (Leonetti et al., 2018). Reactivation of ERK signaling, which may occur in upstream or downstream of the BRAF kinase, representing a major mechanism involved in secondary resistance to BRAF inhibitors. The specific mechanisms include BRAF splice variants (16%), BRAF gene amplification (13%), the increased level of BRAF-V600E homodimers, or secondary mutations in other genes in the MAPK/ERK signaling pathway, which results in BRAF-independent reactivation of ERK signaling. Examples of secondary mutations include neuroblastoma RAS viral oncogene homolog (NRAS)/KRAS mutations (20%) and MEK 1/2 mutations (7%) (Leonetti et al., 2018).

2.3.3 MET alteration

The mesenchymal-epithelial transition factor (MET) dysregulation also manifests as MET exon 14 (METex14) mutation, which is found in 3-6% of lung adenocarcinomas (Park et al., 2015; Suzawa et al., 2019). Other mutations have been reported in the literature, yielding outcomes similar to

those of METex14 mutations. Amino acid substitutions at Y1003 or mutations/deletions in METex14 or its flanking introns provoke an in-frame skipping of METex14 (Park et al., 2015). Although a variety of MET TKIs are being actively developed and used in the treatment of MET-driven NSCLC, the mechanisms underlying acquired resistance to MET-TKIs have not been elucidated. Tepotinib (EMD1214063, MSC2156119J; Merck) is a potent and selective ATP-competitive c-MET inhibitor, specifically targeting tumors with MET exon 14 skipping mutation. The combination of Tepotinib and Gefitinib is also being investigated in the Phase II VISION study, showing increased response rate comparing with chemotherapy group in patients harboring different MET alternations (Park et al., 2015). The known resistance mechanisms to MET inhibitors are mainly different secondary mutations in the MET tyrosine kinase domain, which interfere with drug-kinase binding (Park et al., 2015). Drug resistance observed in crizotinib-treated patients with METex14 mutations and crizotinib-treated patients with increased MET copy number may be caused by second-site MET-Y1230C and MET-D1228N mutations (Bahcall et al., 2018). Increased copy number of the WT KRAS allele is detected in patients with crizotinib-resistant METex14 mutation-positive NSCLC (Bahcall et al., 2018). The mechanisms of drug resistance include: (a) PI3K activation-mediated compensatory induction of dual MET/MEK inhibition, (b) KRAS-induced EGFR ligands expression, and (c) MET-dependent inhibition of PI3K signaling. Suzawa et al. reported that 5 of 113 patients with the METex14 mutation developed KRAS mutations (Suzawa et al., 2019). Of these, one acquired a KRAS mutation after crizotinib treatment, while the other four patients carried KRAS mutations prior to MET TKI treatment. Gene set enrichment analysis (GSEA) of transcriptome data revealed preferential activation of the KRAS pathway, moreover, oncogenic KRAS expression enhanced MET expression. Further

analyses revealed that KRAS mutations also lead to constitutive activation of RAS/ERK signaling and resistance to MET inhibition (Suzawa et al., 2019).

2.3.4 KRAS mutation

The RAS/mitogen-activated protein kinase (MAPK) pathway is another important signaling pathway (Figure 1). KRAS is a member of the RAS protein family and KRAS mutation is a common type of driver gene mutation in lung cancer (Rotow and Bivona, 2017). KRAS mutations occur in 20-30% of patients with NSCLC (Rotow and Bivona, 2017). No effective KRAS inhibitor has been developed to date. Patients with advanced KRAS-positive NSCLC have been treated with MET inhibitors, which fail to significantly prolong survival (Kim et al., 2016; Manchado et al., 2016; Tolcher et al., 2015). The mechanism of resistance may involve activation of the PI3K or fibroblast growth factor receptor 1 (FGFR1) bypass pathways and activation of the receptor tyrosine kinase (RTK)/RAS/ERK signaling pathways (Lin et al., 2015; Rotow and Bivona, 2017). KRAS-mutant tumors are also insensitive to the inhibition of upstream growth factor receptor signaling (Rotow and Bivona, 2017). Therefore, anti-EGFR antibody therapy is ineffective against KRAS-positive NSCLC (Rotow and Bivona, 2017). Patients with KRAS mutations also showed insensitivity to EGFR TKIs (Pao et al., 2005; Rotow and Bivona, 2017). Although KRAS mutations have not been detected in patients with secondary drug resistance, BRAF protein mutations mediate the development of EGFR TKI resistance (Ohashi et al., 2012).

2.3.5 HER2 mutation

HER2 mutations occur in approximately 3% of patients with lung adenocarcinoma (Mazieres et al., 2013). In HER2 mutations, 96% are kinase-activating exon 20 insertion mutations (Arcila et al., 2012). Simultaneous mutations in HER2 and EGFR exon 20 are observed in about 4% of all patients with NSCLC. Mutations in exon 20 alter protein structure and render the TKIs ineffective (Kosaka et al., 2017; Robichaux et al., 2018). Recent data showed that TKIs (afatinib, lapatinib, neratinib, and dacomitinib) exert limited therapeutic effects in patients with HER2-mutated tumors (Kim et al., 2018; Kosaka et al., 2017; Robichaux et al., 2018) and are associated with ORRs of less than 40%. In a therapeutic study of 101 patients with advanced NSCLC, the ORR of patients with HER2 mutations administered a combination of trastuzumab and chemotherapy was approximately 50.9%, while the ORR of patients undergoing chemotherapy alone was 43.5% (Cappuzzo et al., 2006; Kosaka et al., 2017). In contrast, patients treated with a HER2 TKI alone had a lower ORR, ranging from 7.4 to 12%. The EGFR and HER2 exon 20 insertion mutations confer resistance to both first and second-generation EGFR TKIs (Cappuzzo et al., 2006; Kosaka et al., 2017). Kosaka et al. (2017) used afatinib to treat patients with NSCLC with the HER2 V777_G778insGSP mutation and obtained a sustained partial response. A second-site mutation in HER2 (C805S) was found to mediate acquired drug resistance in drug-sensitive HER2 exon 20 insertion models (Robichaux et al., 2018). The second-site PIK3CA mutation and HER2 copy number gain may also lead to secondary resistance (Robichaux et al., 2018).

2.3.6 Rearranged during transfection (RET)

Gene rearrangement involving RET was also observed in approximately 1-2% of patients with NSCLC and mutually exclusive with other oncogenic driver mutations (such as ALK or ROS1

rearrangement or EGFR mutation) (Farago and Azzoli, 2017). Reported common fusion partners are the kinesin family 5B gene (KIF5B) (72%), CCDC6 (23%), NCOA4 (2%), EPHA5 (1%), and PICALM (1%) (Farago and Azzoli, 2017; Lipson et al., 2012). The proportion of RET rearrangement in patients with NSCLC was similar in male and female patients and 63% of patients were non-smokers, 24% were former smokers, and 10% were current smokers (Farago and Azzoli, 2017). Recently, several studies have also identified RET fusion in drug-resistant EGFR mutant NSCLC (osimertinib and other EGFR TKIs). CCDC6-RET was detected by biopsy of patients with osimertinib resistance, however, the potential effect of RET targeting inhibitors are still at the preliminary stage of research. To date, there are no approved drugs for RET rearrangement and treatment remains limited to multi-kinase inhibitors (MKIs), which are associated with significant off-target toxicity and lower efficacy (Yoh et al., 2017). Multi-targeted kinase inhibitors are commonly used in clinical practice to treat RET rearrangement NSCLC, including vandetanib, lenvatinib, sunitinib, sorafenib, alectinib, nintedanib, ponatinib, and regorafenib. However, few cases have been reported and the treatment standard is not clear (Farago and Azzoli, 2017). RET targeted therapy was first reported in 2013, and three of the patients with NSCLC with RET rearrangement were treated with cabozantinib. According to RECIST 1.1, two of the patients had partial responses, and the other three received good treatment outcome, and the survival period was significantly extended (Drilon et al., 2016). A Phase 2 clinical trial evaluating the efficacy of cabozantinib in patients with RET rearranged lung adenocarcinoma is ongoing (Farago and Azzoli, 2017). To date, cabozantinib is still the most clinically reported RET rearrangement targeted therapeutic inhibitor. A study by Piotrowska reported that RET inhibitor (BLU-667) is an effective targeting RET inhibitor, with a long-time

clinical response in patients with RET mutations (Piotrowska et al., 2018). Although there is limited understanding of the mechanisms of acquired resistance to RET inhibitors, repeat biopsy or analysis of circulating tumor DNA are considered promising lines of enquiry.

2.3.7 Neurotrophic tyrosine receptor kinase (NTRK)

NTRK1, NTRK2 and NTRK3 genes encode the neurotrophic tropomyosin receptor kinases (NTRKs), TRKA, TRKB and TRKC, respectively (Klein et al., 1991; Kaplan et al., 1991). TRK proteins act as the receptors of nerve growth factors during normal physiology. The signals are transmitted through PI3 kinase, RAS / MAPK / ERK and PLC-γ pathways, mediating neuronal development. The oncogenic NTRK1 fusions was first reported in NSCLC in 2013. The frequency of NTRK fusions in NSCLC has not been determined, although it is estimated to be 0.1% in all cases of NSCLC, and approximately 3% in patients without other identified driver mutations. Larotrectinib (LOXO-101) is the FDA-approved oral tyrosine kinase inhibitor for tumors with NTRK gene fusions. During phase 1 clinical trial of Larotrectinib, 59 patients were recruited, and 8 carried NTRK gene fusions. The drug is generally well tolerated, and the most common reported adverse event (AE) is fatigue (37% of cases), with anemia reported as the most common grade 3/4 AE (8% of cases). According to the RECIST evaluation, 6 of the 7 patients exhibited a partial response, and one patient was assessed as having stable disease. Larotrectinib is currently being evaluated in a Phase 2 study of NTRK-fusion positive solid tumors (NCT02576431). Entrectinib (RXDX-101) is another oral tyrosine kinase inhibitor with therapeutic effects on ROS1 and ALK, as well as on TRKA, TRKB, and TRKC. Entrectinib was administered to a patient with NSCLC and brain metastases in a Phase 1 clinical trial and is currently being evaluated in the Global Phase 2

Basket Study (Drilon et al., 2017). The patient had a solid tumor carrying NTRK1/2/3, ROS1 or ALK gene rearrangements (NCT02568267). Although NTRK rearrangement tumors are highly sensitive to targeted TRK inhibitors, several cases of acquired resistance to TRK inhibitors have also been reported (Berger et al., 2018). In these cases, patients initially responded well to TRK inhibitors, but then progressed to drug resistance. The potential mechanism is that drug-resistant subclones have new mutations in the NTRK kinase domain, altered protein structure sterically interferes with drug binding, and drug resistance ensues.

Table 1. Acquired resistance in EGFR and ALK-positive NSCLC treated with TKIs[a]

Category	Resistance mechanism	Frequency (%) before TKIs	Currently available drug(s)	Ref.
Resistance to EGFR-TKI	EGFR mutations	10-15%		
	EGFR target mutations (L858R, Del19, T790M, and Ins20)	~60%	Erlotinib and Gefitinib	Sun et al., 2018
	T790M	~50%	Afatinib, Brigatinib and Osimertinib	Sun et al., 2018; Westover et al., 2018
	D761Y, T854A, L747S, C797G, L798I, L718Q, L844V, and L797S	1-2%	Osimertinib and Rociletinib	Minari et al., 2016
	EGFR	Several	Osimertinib and Rociletinib	Minari et al.,

	amplification	cases reported		2016
	C797S or loss of EGFR-T790M^{-}	Several cases reported	Osimertinib	Ortiz-Cuaran et al., 2016; Rotow and Bivona, 2017
	Bypass signaling tracks	~20%		Camidge et al., 2014
	BRAF mutations	1%	Vemurafenib, Dabrafenib + Trametinib combination	Planchard et al., 2016
	ROS1 rearrangements	2%	Crizotinib/Ceritinib/Loratinib	Roskoski, 2017
	RET rearrangement	1-2%	Cabozantinib/Vandetanib/Alectinib	Anna et al., 2017
	NTRK fusion	3%	Larotrectinib/Entrectinib	Anna et al., 2017
	MET amplification	5%	Gefitinib, Crizotinib, Capmatinib	Bahcall et al., 2018; Cappuzzo et al., 2009

	HER2 amplification	8-13%	Neratinib + Temsirolimus/Afatinib/Dacomitinib or Neratinib alone	Camidge et al., 2014; Robichaux et al., 2018
	PIK3CA mutation	2-3%		
	KRAS mutations	3%		
	Phenotypic changes	3-10%		Westover et al., 2018
	Transformation to SCLC (CDKN2A loss, MTOR mutations and FGFR3 alterations)	3-10%	Platinum + EGFR-TKIs	Ortiz-Cuaran et al., 2016
	Unknown mechanism	10-20%		Westover et al., 2018
Resistance to ALK-TKI	ALK mutations	3-7%		
	ALK target	50%	Crizotinib	Crystal and

	mutations			Shaw, 2011; Solomon et al., 2014
	Secondary mutations in ALK (L1196M, G1269A, G1202R, F1174L and V1180L, et al)	~33%	Ceritinib, Alectinib, and Brigatinib	Dong et al., 2016; Friboulet et al., 2014a; Qian et al., 2017; Spaans and Goss, 2014
	ALK G1202A	Several cases	Repotrectinib	Drilon et al. 2018
	ALK amplification	~16%	Crizotinib, Ceritinib, and Alectinib	Doebele et al., 2012; Katayama et al., 2012
	Bypass signaling tracks	~35%		
	Increased EGFR signaling	Up to 30%	Brigatinib	Khan et al., 2018

	c-KIT amplification and SCF overexpression	~10%	N/A	Qian et al., 2017
	IGF-1R activation	4 or 5 cases	N/A	Wilson et al., 2017
	HGF and MET activation	one case reported (for alectinib)	Alectinib versus Crizotinib	Huang, 2018
	Phenotypic changes			
	Transformation to SCLC	< 10 (cases reported)		Qian et al., 2017
	Unknown mechanism	~15%		Camidge et al., 2014; Qian et al., 2017
KRAS mutations	KRAS mutations	20-30%	Abemaciclib (CDK4/6) or Erlotinib (EGFR)	Pao et al., 2005; Rotow

				and Bivona, 2017
Unknown	Unknown mutations	~40%		

N/A: not available

[a] Only mechanisms clinically identified in patients are shown.

3. Epigenetic alterations and drug resistance

Genetic alterations in cancer include driver mutations, closely related to tumorigenesis, as well as other mutations with an indirect correlation with tumor formation and progression (Rotow and Bivona, 2017). Studies have established that these changes are related to TKIs drug resistance (Table 1). A topical area of current research into indirect mutations concerns epigenetic alterations (Jones et al., 2016; Schiffmann et al., 2016). Epigenetic alterations mainly include promoter DNA hypermethylation, hypomethylation of whole genome DNA, histone acetylation, and abnormal chromatin structure. In addition, noncoding genes, such as long noncoding RNA (lncRNA) or microRNA (miRNA), have also been found to regulate the expression level of proteins associated with cell apoptosis and epithelial-mesenchymal transition (EMT), further modulating the effect of TKIs in lung cancer treatment (Jones et al., 2016; Schiffmann et al., 2016).

3.1 DNA methylation

Cancer cells typically exhibit abnormal profile of DNA methylation characterized by

promoter hypermethylation and hypomethylation of whole genome DNA (Vendetti and Rudin, 2013). Whole-genome hypomethylation can promote tumor formation (Esteller, 2007; Jones and Baylin, 2002), and studies have shown that hypomethylation of the whole genome leads to genomic instability and abnormal activation of oncogenes or transposable elements (Daskalos et al., 2009; Ehrlich, 2009). In contrast, promoter hypermethylation can induce selective inhibition of gene expression, such as inhibition of tumor suppressor gene expression. During tumorigenesis, promoter hypermethylation can result from different mechanisms, such as loss-of-function or mutations of genes encoding DNA demethylases (TET1, TET2, and TET3) or overexpression of genes encoding DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B) (Jones and Liang, 2009; Rhee et al., 2002). DNA methyltransferase (DNMT) transfers a methyl group to cytosine using S-adenosylmethionine as the methyl donor. Three types of DNMTs are overexpressed in NSCLC (Lin et al., 2010; Tang et al., 2012), and overexpression of DNMT1 is closely correlated with poor prognosis. Furthermore, whole genome hypomethylation underlies the genomic instability caused by a mutation in or loss-of-function of DNMT. To date, the FDA has approved three DNMT inhibitors (DNMTi), Azacitidine (Vidaza), Decitabine (Dacogen), and Guadecitabine (Table 2) (Vendetti and Rudin, 2013).

Several studies have elucidated the epigenetic alterations in patients with lung cancer. The DNA hypermethylation profiles of the promoter sequences of transcription factor genes have also been identified (Park and Han, 2019). Studies reported homeobox (HOX)-related genes, including MSX1, IRX2, PAX6, SIX, LHX, DLX, CDKN2A, MLH1, MSH2, APC, RARB, MGMT, and ENGRAILED, as well as the HOXA cluster genes

HOXA7 and HOXA9 (Chang et al., 2017; Fang et al., 2018; Plowright et al., 2009). Plowright et al. (2009) suggested that HOX gene transcription factors were biomarkers for the early diagnosis and/or monitoring of therapeutic outcomes in patients with lung cancer. Indeed, high expression level of mesenchyme-HOX2 (MEOX2) is associated with drug resistance, prognosis, and survival rate in patients with lung cancer (Avila-Moreno et al., 2014; 2016). Drugs that target epigenetic alterations generally induce cell apoptosis or differentiation by reversing the abnormal gene silencing or activation (Schiffmann et al., 2016). These types of drugs have much lower cytotoxicity and fewer side effects than conventional chemotherapeutic drugs. Sandoval et al. (2013) confirmed that such drugs are more appropriate for patients with high-risk NSCLC who are not suitable for chemotherapy, or with a short survival period, and show no recurrence. However at present, drugs that target epigenetic alterations in lung cancer are mainly used in combination with other therapeutic drugs, and are still under the pre-clinical trial stage (Rotow and Bivona, 2017). Li et al. (2013) found a correlation between DNA methylation of the EGFR gene promoter and TKI resistance in NSCLC cells. Decitabine, a DNMT inhibitor, can enhance or even restore sensitivity to gefitinib, resulting in growth inhibition and apoptosis in tumor cells and decreased EGFR protein expression (Li et al., 2013). Bauman et al. also showed that a combination of azacitidine and erlotinib was well tolerated (Bauman et al., 2012).

3.2 Histone acetylation

Enzymes responsible for histone acetylation are histone acetyltransferase (HAT) and

histone deacetylase (HDAC) (Vendetti and Rudin, 2013). Histone acetylation can be accomplished by the loss of HAT function or the upregulation of HDAC expression, although the underlying mechanisms remains unclear (Vendetti and Rudin, 2013). Studies indicated that HDAC inhibitors can reactivate the transcription of tumor suppressor genes, such as P21 (also known as CDKN1A), by increasing histone acetylation (Marks, 2010; Rosato and Grant, 2003). Further studies reported that HDAC can induce the deacetylation of nonhistone proteins, such as p53 and NF-κB (Schafer et al., 2017). HDAC inhibitors can regulate the stability or activity of many key transcription factors. Through a variety of mechanisms, HDAC inhibitors can exert anti-tumor effects by inducing cell apoptosis and cell cycle arrest (Valente et al., 2013). Currently, there are four different types of HDAC inhibitors with distinct structures; of these, three have been approved, including vorinostat, belinostat, chidamide, and romidepsin (Table 2), used for the treatment of cutaneous and peripheral T-cell lymphoma; while panobinostat, used for the treatment of multiple myeloma (Rosato and Grant, 2003). Sharma et al. (2013) reported that HDAC inhibitors can effectively kill resistant cells after EGFR inhibitor treatment. HDAC inhibitors may also alter the activity of enhancers, key DNA elements that regulate the expression of specific genes in cells, thereby regulating the transcription of genes involved in drug responses (Sharma et al., 2013). Many studies have confirmed that epigenetic alterations are associated with EGFR TKI resistance and that drug resistance characteristics are gradually induced during the initial stage of EGFR TKI treatment (Cheng and Chen, 2014). For example, HDAC can promote the survival of EGFR TKI-resistant cells, however, a combination of erlotinib and the HDAC inhibitor

panobinostat/vorinostat can improve therapeutic effect *in vitro* (Greve et al., 2015; Wang et al., 2018). Other clinical trials using different combinations of EGFR TKIs with HDAC inhibitors are currently ongoing (Gerber et al., 2015). Preclinical studies reported that the HDAC inhibitor romidepsin can enhance the efficacy of erlotinib in *in vitro* treatment of NSCLC (Gerber et al., 2015). The HDAC inhibitor entinostat (SNDX-275) combined with gefitinib/erlotinib also obtained great inhibitory effect on TKI-resistant NSCLC cells (Juergens et al., 2011; Li et al., 2017; Ruiz et al., 2015).

3.3 Histone methylation

Histone lysine methyltransferases (KMT) are responsible for histone methylation, while histone lysine demethylases (KDM), such as LSD-1 and 2, or histone demethylase containing a Jumonji domain-containing proteins (JmJD) domain, are responsible for demethylation regulation (Pirola et al., 2018; Wilson, 2007). The monomethylated lysine or the dimethylated lysine at position 4 and 9 on histone H3 are demethylated by LSD1. Studies indicated that LSD1 is usually overexpressed in NSCLC and promotes tumor cell proliferation and invasion (Lv et al., 2012). When targeting LSD1 in hematological tumors, LSD1 inhibitors can block the differentiation of acute myelogenous leukemia (AML) cells and reverse the sensitivity of AML cells to all-trans retinoic acid (ATRA) (Sugino et al., 2017). The first LSD1 inhibitor used in a clinical setting was a tranylcypromine compound, a monoamine oxidase inhibitor approved for the treatment of complex depression 50 years ago (Maslinska, 1984). At present, only a few studies on histone demethylation in NSCLC have been conducted. Specific LSD1 inhibitors are still in preclinical and early

clinical development stages (Abdel-Magid, 2017; Stazi et al., 2016). In addition, lysine 27 methylation on histone H3 (H3K27) is regulated by the enhancer of zeste homolog 2 (EZH2). EZH2 is overexpressed in many kinds of tumors, including NSCLC. The 3-Deazaneplanocin A (DZNep) is an EZH2 inhibitor that can decrease H3K27 methylation level and restore abnormally silenced genes in NSLC cells (Kikuchi et al., 2012).

3.4 Chromosomal heterogeneity

The chromosome is mainly composed of DNA and nucleosomes, with DNA strands twisting around histone core proteins to form nucleosomes, and these provide secondary structure and affect DNA repair, replication, transcription and mitosis. The mating type switching (SWI)/sucrose nonfermenting (SNF) chromatin remodeling complex regulates gene expression and changes chromatin structure in an ATP-dependent manner (Song et al., 2014). Gene mutations involving nucleosomes include mutations in the components of the SWI/SNF complex, such as ARID1A, ARID1B, BRG1 (also known as SMARCA4), SNF5 (also known as SMARCB1), PBRM1, and the chromodomain helicase DNA binding (CHD) protein family. These mutations can interfere with gene transcription involved in controlling cell proliferation and fate determination and promote cancer development by disrupting DNA repair (Song et al., 2014). Song et al. also reported that BRG1 (an ATPase subunit) deficiency promotes NSCLC progression. Different types of chromatin modifications can be used as diagnostic prognosis indicators (Roche et al., 2013; Song et al., 2012), for example, abnormal expression of HDAC or abnormal methylation of H3 and H4 are correlated with poor prognosis.

Table 2. Approved epigenome-targeting drugs or in clinical trials for metastatic NSCLC treatment

Epigenetic therapy	Drug	Target	Mechanism	Approval or trial status		Ref.
				Status	Clinical NSCLC trials	
DNMT inhibitors	Azacitidine (Vidaza)	Pan-DNMT	Inhibition of DNA methylation	FDA	Alone (Phase II study)	Holoye et al., 1987
	Decitabine (Dacogen)	Pan-DNMT		FDA	Decitabine + Valproic (Phase I study); alone (pilot Phase I-II study)	Chu et al., 2013; Momparler et al., 1997
HDAC inhibitors	Belinostat (Beleodaq)	HDAC class I and class II	Reduces oncogene transcription	FDA	Seliciclib + Belinostat (in vivo and in vitro)	Ong et al., 2016
	Panobinostat (Farydak)	HDAC class I,		FDA	Panobinostat + radiotherapy/chemora	Takhar et al.,

		class II, and class IV	and signaling, and promotes cell cycle arrest and apoptosis		diotherapy (Phase I study)	2015
	Vorinostat (Zolinza)	HDAC class I, class II, and class IV		FDA	Erlotinib/Gefitinib + Vorinostat (Phase I/II study)	Han et al., 2015
	Romidepsin (Istodax)	HDAC class I		FDA	Erlotinib + Romidepsin (Phase I study)	Gerber et al., 2015
	Entinostat (Sndx-275)	HDAC class I		Phase III study	Erlotinib + SNDX-275 Phase II study	Ruiz et al., 2015
BET inhibitors	TEN-010 (RO6870810/RG6146/JQ2)	Pan-BET	Inhibition of BET binding to acetylated	Phase I study	Phase I study	Jones et al., 2016

			histones			
	I-BET762 (GSK525762)	Pan-BET		Phase I/II study	Phase I study	Jones et al., 2016
	OTX105 (MK-8628)	Pan-BET		Phase I/II study	Phase I study	Chen and Li., 2017
	ABBV-075	Pan-BET		Phase I study	Phase I study	Chen and Li., 2017

3.5 Noncoding RNA

Although the genetic mechanisms underlying acquired resistance to targeted therapies have been widely studied, very few studies invovling epigenetic alterations in noncoding genomes and drug resistance have been reported. Non-coding RNA refers to a functional RNA molecule that cannot be translated into a protein. Long non-coding RNA (LncRNA) maks up a group of noncoding RNAs composed of more than 200 nucleotides with low or

nonprotein-coding functions (Wei and Zhou, 2016). Accumulating evidences suggest that aberrant expression of lncRNA contributes to a variety of biological functions underlying transcriptional and posttranscriptional regulation and is closely associated with the resistant occurrence (Wei and Zhou, 2016). Wei and Zhou also demonstrated the abnormal expression of lncRNA CAR10 in lung cancer, which is associated with air pollution. Notably, a number of dysregulated lncRNAs, such as HOTTIP, BLACAT1, and SOX2/ANRIL, have also been identified in the serum of patients with NSCLC (Table 3) (Huang et al., 2019; Navarro et al., 2019; Xie et al., 2018). Studies have shown that this type of lncRNA can be used as diagnostic markers for screening NSCLC by peripheral blood testing. lncRNAs can also be used as prognostic markers for patients with NSCLC; for example, expression level of lncRNA GPR158-AS1 is inversely correlated with the OS rate of patients with NSCLC (Xie et al., 2018). In contrast, lncRNA RP11-94L15.2 and AC104134.2 expression levels are positively correlated with OS rate (Wei and Zhou, 2016).

Micro RNAs (miRNAs) are relatively small endogenous noncoding RNA molecules that inhibit the expression of regulatory genes and affect cell behavior through translation. In addition to their roles in promoting or inhibiting tumor growth, miRNAs also play an important role in regulating the functions of resistance-related genes (Table 3). For example, the miR-29 family members can directly down regulate DNMT3a and DNMT3b, and indirectly down regulate DNMT1 through interaction with the 3'-untranslated region of Sp1 (El-Awady et al., 2015). Reduced miR-29 expression is associated with increased

expression of DNMT3a and DNMT3b in NSCLC. Yun et al. (2018) reported that ALK inhibitors are effective in the treatment of ALK fusion gene-positive lung cancer, but always associated with acquired drug resistance. The acetylation of histone H3 lysine 27 (H3K27ac) undergoes significant changes during drug resistance acquisition, which decreases H3K27ac level and miR-34a expression associating with activation of target genes, such as AXL. Panobinostat can alter the H3K27ac pattern, activate the tumor suppressor genes miR-449 and miR-349, and synergistically induce ALK inhibitors to antagonize drug-resistant cells (Yun et al., 2018).

Table 3. The ncRNAs involved in NSCLC: expression level and functions

ncRNAs	Expression	EGFR-TKIs	Biomarker (B) or resistance factor (R)	Study type	Associated signaling cascade(s) and/or transcription factors	Refs.
LncRNAs						
SOX2/ANRIL	Up	N/A	B	Human samples	N/A	Xie et al., 2018
HOTTIP	Up	N/A	B	Human	N/A	Navarro et al.,

				samples		2019
HOTAIR	Up	N/A	B	Human samples	N/A	Li et al., 2017
GAS5	Down	Geftinib/Cisplatin	R/B	*In vitro* and human samples	EGFR/PI3K/AKT	Dong et al., 2015
AK126698	Down	Cisplatin	R	*In vitro*	N/A	Yang et al., 2013
BLACAT1	Up	Cisplatin	R	*In vitro*	Autophagy	Huang et al., 2019
NEAT1	Up	Paclitaxel	R	*In vitro*	Akt/mTOR	Li et al., 2019
miRNAs						
miRNA-21	Up	N/A	B	*In vitro* and human	MAPK, AP-1, NFIB, RASV12, ID-1	Fujita et al., 2008

				samples		
miRNA-7	Down	N/A	B	Human samples	Ras/ERK/Myc, EGFR	Chou et al., 2010
miRNA-34a and miRNA-199a/b	Down	N/A	B	Human samples	Axl receptor/tyrosine kinase, P53, ELK1, ERK-MAPK	Muddaluru et al., 2011
miRNA-21/23b	Up	Erlotinib, Vandetanib	R	*In vitro*	EGFR	Sarkar et al., 2010
miRNA-34a and miR-449a	Down	Crizotinib, Ceritinib	R	Human samples	AXL	Yun et al., 2018
miRNA-221	Up	TRAIL	R	*In vitro*	AKT pathway and metallopeptidases	Garofalo et al., 2009

miRNA-222	Down	Geftinib	R	*In vitro*	EGF/MET	Zucali et al., 2008
miRNA-145	Up	Geftinib	R	Human samples	c-Myc/eIF4E pathway, EGFR receptor	Chen et al., 2010; Zhong et al., 2010
miRNA-126	Up	Geftinib	R	*In vitro* and human samples	EGFL7 and VEGF-A	Donnem et al., 2011
miRNA-214	Up	Geftinib	R	*In vitro*	PTEN/AKT	Wang et al., 2012
miRNA-128	Down	Geftinib	R	Human samples	EGFR	Weiss et al., 2008

4. Tumor microenvironment heterogeneity and drug resistance

Tumor cells rely on a specific environment for survival and proliferation, which is defined as tumor microenvironment (TME) (Figure 2). TME consists of tumor cells, resident or

infiltrating nontumor cells that mutually interact with tumor cells, as well as noncellular components. Nontumor cells include activated fibroblasts, vascular and lymphatic endothelial cells, immune cells, and pericytes, while noncellular components include the extracellular matrix (ECM) and a variety of soluble factors (Maman and Witz, 2018). Tumor heterogeneity poses complex challenges to preclinical and clinical studies. Tumor growth and metastasis depends on changes in the genetic characteristics of tumor cells but is also affected by the adaptive advantages of mutations conferred in a particular environment (Junttila and de Sauvage, 2013). Tumor formation and development is a dynamic process (Binnewies et al., 2018) and tumor heterogeneity accounts for the mechanisms of drug resistance.

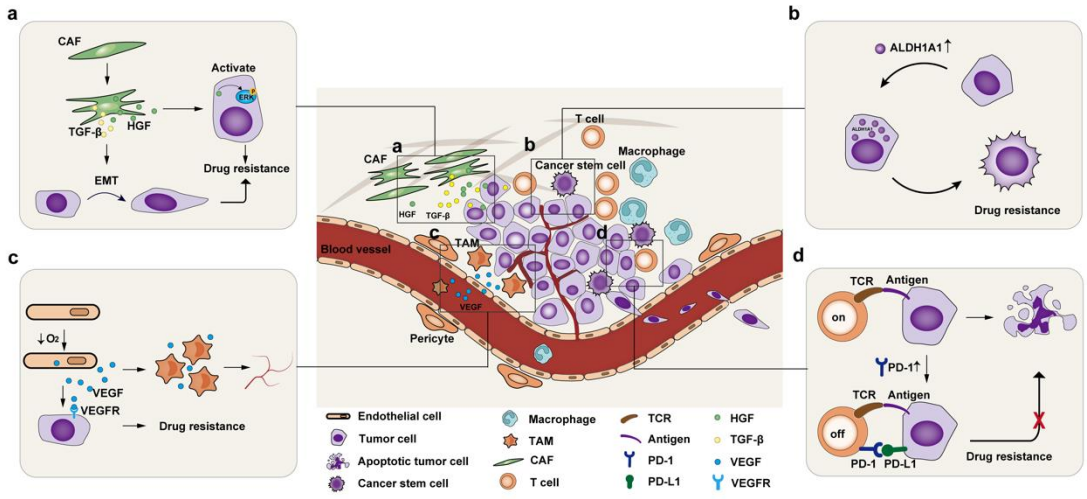

Figure 2 Tumor microenvironment heterogeneity and drug resistance

4.1 Drug resistance induced by cancer-associated fibroblast

Cancer-associated fibroblasts **(**CAFs) are a type of activated fibroblast, which are a component of the TME and play prominent roles in tumorigenesis and progression. Currently, fibroblasts are considered to be mesenchyme-derived cells, whose main function is to maintain the structural framework of the tissue. Under normal physiological

conditions, quiescent fibroblasts are activated to respond to tissue injury and support repair functions. Under pathological conditions, such as in tumor tissue, fibroblasts secrete several matrix metalloproteinases (MMPs), MMP inhibitors, and other types of cytokines, which are released into the extracellular space and specifically alter the surrounding TME, to inhibit tumor development (Polanska and Orimo, 2013). However, tumor-associated CAFs can also express and secrete different growth factors and proteases to promote tumor progression (Koczorowska et al., 2017; Yi et al., 2018; Yu et al., 2014). At present, only a few studies have focused on understanding CAF mechanisms in developing drug resistance (Choe et al., 2015; Kim et al., 2013; Shintani et al., 2016). Studies have shown that co-culturing CAFs with cancer cells can induce EMT and *in vitro* resistance to EGFR TKIs in NSCLC cells (Yi et al., 2018). Multiple paracrine factors secreted by CAFs including HGF, can promote ERK activation, subsequently causing NSCLC tumor cells to become resistant to EGFR TKI (Kanaji et al., 2017; Siegfried et al., 1997; Yi et al., 2018). Therefore, co-treatment with HGF-targeting drugs restores NSCLC sensitivity to EGFR TKI.

CAFs can also enhance ECM deposition, increase secretion of inflammatory factors, stimulate vascular permeability to prevent drug accumulation, and promote drug resistance (Choe et al., 2015). In a study of co-cultured lung cancer cells, podoplanin-expressing CAFs exhibited a driving effect on the development of EGFR-TKI resistance, although the underlying molecular mechanism remains unclear (Kolli-Bouhafs et al., 2012). Interestingly, therapeutic responses were observed when target CAF with focal adhesion kinase (FAK) inhibitors, indicating that fibroblast-targeting strategies can

partially overcome cancer cell resistance to targeted therapies (Kolli-Bouhafs et al., 2012). At present, the FDA has not approved drugs that directly target CAFs in patients with NSCLC. The drugs currently being evaluated are mainly target MMPs, secreted by CAFs, and most of these are at the Phase III clinical trials stage. AE-941, also known as Neovastat, is a standard water-soluble shark cartilage extract (Gingras et al., 2001). *In vitro* studies demonstrated that AE-941 specifically inhibits vascular endothelial growth factor (VEGF)-based signaling pathways, and several of matrix metalloproteinase (MMPs) activities, including MMP-2, MMP-9, and MMP-12. However, AE-941 combined with chemotherapy or radiation therapy did not improve the OS rate in patients with stage III NSCLC (NCT00005838) (Lu et al., 2010). Rebimastat also targets MMP-1, MMP-2, MMP-8, MMP-9, and MMP-14 in patients with stage III NSCLC, but clinical trials have not been successful (NCT00006229) (Junttila and de Sauvage, 2013).

4.2 Drug resistance induced by vascular and lymphatic endothelial cells

Several types of tumor-associated endothelial cells, such as tumor vascular endothelial cells, also play important roles in tumor development (Kalluri, 2003). A subset of tumor vascular endothelial cells is derived from the abnormal proliferation of normal endothelial cells, such as the new blood vessels that continuously form at the site of original normal blood vessels, or the endothelial progenitor cells that originate from the bone marrow. These abnormal cells form additional tumor-related blood vessels for tumor cells to assimilate sufficient nutrition to support their unlimited proliferation (Kalluri, 2003). Tumor cells are provided with rich nutrients through structural changes in the existing vascular

system, such as the formation of complex branch vessels or uneven blood vessels that constitute a dynamic and heterogeneous vascular network system (Ferrara and Kerbel, 2005; Junttila and de Sauvage, 2013). In addition, when the balance between proteases and inhibitors is disrupted, tumor-related vascular endothelial cells also express proteases and inhibitors to remodel microenvironment for tumor growth (Binnewies et al., 2018). Tumor vasculature is inhomogeneous, tortuous, and highly permeable, which make it more complex, leading to tumor tissue heterogeneity, and ultimately affecting clinical treatment (Forster et al., 2017). For example, the formation of new blood vessels causes damage to the blood supply, leading to impaired delivery of anti-cancer drugs (Melincovici et al., 2018). Local secretion of VEGF, which responds to hypoxia, can promote angiogenesis. It can also affect the efficacy of EGFR TKI and lead to the development of drug resistance in patients by acting in a feed-forward manner to promote VEGF and VEGF receptor (VEGFR) binding in tumor cells and initiate downstream signaling pathways (Passaro et al., 2016; Seki et al., 2019). Therefore, the VEGF and VEGFR signaling pathways have always been considered hot targets in anti-angiogenesis studies, and anti-tumor effects can be achieved by inhibiting these pathways (Table 4).

In 2004, the VEGF A-targeting, monoclonal antibody bevacizumab was approved by the FDA for NSCLC treatment, although the efficacy of using the antibody alone was not satisfactory and tumor growth in patients was not well controlled (Hall et al., 2015). When bevacizumab was used in a mouse model of lung cancer, it resulted in acquired drug resistance, which was partially acquired by the upregulation of VEGFA, FGF2, FGFR2, and PDGFRα in stromal cells (Hall et al., 2015). Moreover, in lung adenocarcinoma

patients, the number of fibroblast-specific protein 1 (FSP1)-positive fibroblasts in tumors treated with bevacizumab was higher than in untreated patients (Russo et al., 2017). However, the combination of bevacizumab with chemotherapy drugs can prolong patient survival, and its drug resistance profile is similar to that of other monoclonal antibodies. VEGF mutations to VEGF C and VEGF D are also observed, which are off-target effects (Russo et al., 2017). Ramucirumab, a recombinant human immunoglobulin G1 monoclonal antibody used as a receptor antagonist, is designed to block the ligand binding site of VEGFR 2, and its combination with docetaxel was evaluated as a second-line treatment in patients with metastatic NSCLC (Garon et al., 2014; Smit et al., 2018). This treatment effectively improved PFS of patients, but ramucirumab resistance developed with the VEGFR-2 mutant cells. Vandetanib is a TKI used for the treatment of late-stage NSCLC (Morabito et al., 2010; Xiao et al., 2013). The treatment of drug resistance is also limited by VEGFR, which targets the catalytic domain of VEGFR. VEGFR TKI did not improve OS when used in monotherapy or in combination with chemotherapy in patients with NSCLC (Morabito et al., 2010). Cascone et al. (2017) reported that the HGF/c-MET pathway mediates VEGFR inhibitor resistance and vascular remodeling in NSCLC. In addition to directly promoting angiogenesis, VEGF can also recruit tumor-associated macrophages (TAM) that secrete VEGF into the TME to indirectly stimulate angiogenesis (Wang et al., 2011). The combination of EGFR TKI with VEGF-targeting inhibitors or monoclonal antibodies is currently undergoing clinical evaluation (Li et al., 2014; Takeuchi et al., 2012).

Pericytes are usually dispersed around capillaries and venous endothelial cells, and are

involved in disease pathogenesis such as cancers (Binnewies et al., 2018). Pericytes exhibit several important functions, such as stabilizing blood vessels, inhibiting excessive proliferation of endothelial cells, maintaining capillary diameter, regulating blood flow, and producing survival signals for endothelial cells, among others. Although many studies have shown that pericytes participate in a variety of pathological processes, the specific mechanisms regulating pericytes and whether pericytes are involved in drug resistance remains unclear.

4.3 Immune system-mediated drug resistance

TME-induced resistance to NSCLC targeted therapy remains a challenging issue, particularly with the emergence of new therapies regulating immune cells. It is still unclear whether there exists a therapeutic synergy between driver gene targeting and immunosuppressive agents and whether there are molecular subtypes of NSCLC.

Recruitment and localization of immune cells into TME varies considerably during disease progression (Fridman et al., 2012). Immune system-based regulatory functions are critical during tumorigenesis and progression, and may affect patient response to clinical drugs (Binnewies et al., 2018; Junttila and de Sauvage, 2013).

The criteria for immune-checkpoint inhibitor (ICI) treatment in patients with metastatic NSCLC, molecular diagnostics, and promising biomarkers and radiological methods for response evaluation, were discussed in the European Society for Medical Oncology Open access journal (ESMO Open) in July 2018 (Berghoff et al., 2019). ICI treatment should only be performed in patients with NSCLC who are not responding to targeted therapy or

in whom other treatments, including chemotherapy, are ineffective (Berghoff et al., 2019). Recent progress has been reported using a combination of targeted therapy and ICI therapy for patients with metastatic NSCLC with improved prognosis of selected patients (Berghoff et al., 2019).

A number of immunomodulators (Table 4) for cancer treatment have recently been approved by the FDA, and markedly improve the applicability of immunotherapy (Pistamaltzian et al., 2019). In clinical tissue specimens, activation of the EGFR pathway induced expression of programmed cell death 1 ligand 1 (PD-L1) and other immunosuppressive factors (Chae et al., 2018). Tumor cells employ programmed cell death 1 (PD-1) to mediate inhibitory signaling transduction and avoid T-cell mediated eradication, which can induce overexpression of their cognate ligand PD-L1 (Chae et al., 2018). Upregulation of the programmed cell death 1 ligand 1 (PD-L1) gene occurs in cells with activated EGFR mutations or ALK rearrangements, resulting in less permissive T cell-mediated anti-tumor cytotoxicity environment. However, inhibitor-based therapy against PD-L1 (or programmed cell death protein 1 (PD-1)) has not been shown to achieve significant efficacy in patients with NSCLC with EGFR mutations or ALK rearrangements, with only one study reporting an ORR of 3.6% (Gainor et al, 2016). The poor efficacy may be due to the low immunogenicity of genomic complex tumors with the specific oncogenic driver mutations, and low level of CD8+ T cell infiltration in tumors were also detected (Gainor et al, 2016). Pembrolizumab is a humanized monoclonal antibody directed against PD-1. Pembrolizumab was approved for first-line treatment in patients with NSCLC with overexpression of PD-L1. A clear improvement in PFS and fewer

adverse events when combined with chemotherapies were observed. A randomized Phase 2 clinical trial reported improved therapeutic effect among patients who had not been administered treatment compared to those undergoing platinum-based chemotherapy. Of note, no activated EGFR mutation or ALK rearrangement were detected in patients in either pembrolizumab-based trial (Reck et al., 2016; Langer et al., 2016). Borghaei, et al. reported the results of a trial involving patients with non-squamous NSCLC. No improvement in survival was obtained amongst patients with EGFR mutations (15% of the total number of patients) after nivolumab treatment, with a hazard ratio (HR) of 1.18. The OS of patients treated with nivolumab was greater (HR 0.75) in all patients receiving treatment (Borghaei et al., 2015). The nivolumab antibody was recently approved for the treatment of patients with metastatic squamous cell lung cancer (Ready et al., 2019). Encouraging clinical effects in selected patients have been reported using nivolumab mediated blockade of the PD-1/PD-L1 interaction (Bylicki et al., 2019; Costantini et al., 2019). Approximately 20% of patients reported a long-lasting partial response and some patients reported long-term survival with an extension of 3.2 months (Costantini et al., 2019; Pistamaltzian et al., 2019). Julien et al. analyzed treatment efficacy of ICI in 527 patients with NSCLC harboring different activating molecular alterations, including 252 cases of KRAS, 110 cases of EGFR, 38 cases of BRAF, 36 cases of MET, 23 cases of HER2, 18 cases of ALK, 14 cases of RET, 5 cases of ROS1, as well as 31 cases of multiple drivers. Patients with KRAS, BRAF, and MET-exon 14 alternations showed a greater response than patients with EGFR, ALK, and RET alternations (Julien et al., 2018). However, the immune escape mechanism of the

PD-1/PD-L1 pathway in specific genomic subpopulations of NSCLC is not clearly understood (Pistamaltzian et al., 2019).

Recent studies have also focused on the interaction between tumor cells and tumor-associated macrophages (TAM). This interaction was reported to affect the survival of tumor cells after EGFR TKI treatment (Pistamaltzian et al., 2019; Schrank et al., 2018). In a mouse model of NSCLC, analysis of differential RNA expression between tumor and normal cells showed that macrophage-derived factors activated a variety of tumor cell signaling pathways, including MAPK, PI3K, YAP, NF-κ, Wnt, and RAS pathways, and were involved in resistance to EGFR inhibitors (Binnewies et al., 2018). Elevated TAM infiltration level in the TME was associated with decreased PFS and OS in patients with late-stage NSCLC carrying EGFR mutation after EGFR TKI treatment (Rakaee et al., 2019; Singhal et al., 2019).

Table 4. Representative drugs targeting tumor microenvironment during NSCLC treatment

Drug name	Molecule type	Target	Indication	Year approved	Drug developer	Status (reference)	Mechanism of drug resistance
Vasculature system							
Bevacizumab	Antibody	VEGFA	Combination with carboplatin/paclitaxel for BM in	2004	Genentech and Roche	FDA approved [(BLA) 125085]	Overexpression of PDGFRB, NRAS

			NSCLC				mutation
Vandetanib	Small molecule	VEGFRs, PDGFRs, EGFR	Combination of dabrafenib and trametinib in NSCLC	2011	AstraZeneca	FDA approved [(NDA) 022405]	Overexpression of PDGFRB, second NRAS mutation
Cabozantinib	Small molecule	VEGFR2, RET, MET, KIT, TRK, FLT3, AXL and TIE2	RET rearrangement or MET amplification in NSCLC with EGFR-TKIs resistance	2012	Exelixis	FDA approved [(NDA) 203756]	None reported
Immune system							
Nivolumab	Antibody	PD1	Combination with ipilimumab for BM in NSCLC	2014	Bristol-Myers Squibb	FDA approved	None reported

Pembrolizumab	Antibody	PD1		2014	Merck	FDA approved	None reported
Atezolizumab	Antibody	PDL1		2016	Genentech and Roche	FDA approved	None reported
Lpilimumab	Antibody	CTLA-4	Combination with carboplatin for BM in NSCLC	2011	Bristol-Myers Squibb	FDA approved [(BLA) 125377]	None reported

4.4 Cancer stem cell-mediated drug resistance

Stem cells are a group of cells with unlimited proliferative ability and multidirectional differentiation potential that exist in tumor tissue and are closely associated with tumor recurrence, metastasis, and resistance to chemotherapy and radiotherapy (Eramo et al., 2008). Tumor cell population with stem cell characteristics can be identified by specific cell surface markers. In NSCLC studies, CD133, c-KIT, and ALDH1A1 are generally used as molecular markers (Levina et al., 2010; Sullivan et al., 2010). In *in vitro* studies, drug-resistant cells were obtained by screening EGFR-mutant lung adenocarcinoma cells using a concentration gradient of drugs-displayed stem cell-like attributes, such as increased expression of ALDH1A1, increased percentage of side population (SP) cells, and enhanced self-renewal ability (Sullivan et al., 2010). Drug-resistant cells also acquired

better tolerance to chemotherapeutic drugs (Shien et al., 2013). In addition, ALDH1A1 protein expression in tumor tissue was also significantly increased after the development of secondary drug resistance (Shien et al., 2013). Honkanen et al. (2017) reported that cancer stem cell-like cell (CSLC)-mediated therapeutic resistance in ALK-rearranged lung cancer represents one of many different mechanisms of ALK TKI resistance.

5. Other therapeutic strategies

Although different mechanisms of drug resistance to targeted therapies have been investigated, clinically there is still no effective treatment for drug-resistant tumors. New drugs targeting gene mutations are being developed, meanwhile current research is also focusing on design multi-drug combinations. Relevant clinical trials and therapeutic strategies are listed in Table 5. The results to date indicate that combination therapies or multi-target drugs can achieve greater efficacy. The related studies are as follows: EGFR-TKI/ALK inhibitor plus monoclonal antibody; EGFR-TKI plus chemotherapy drugs; EGFR-TKI/ALK inhibitor plus bypass pathway inhibitor; as well as EGFR-TKI plus HDAC inhibitor. As shown in Table 5, these treatments only extended PFS by two or three months, and some combination drugs exhibited certain effects on patients with drug resistance.

Immunotherapy has been extensively investigated in recent years, although multifunctional drugs have also been proposed in many preclinical studies, such as antibody-drug conjugates (ADCs) or other similar drug delivery systems (Abdollahpour-Alitappeh et al., 2019; Zhuang et al., 2019). ADCs selectively deliver toxic

drugs to tumor cells or their surrounding microenvironment through the binding of specific cell surface molecules on tumor cells, increasing local drug concentration and targeted drug release, resulting in more effective therapeutic outcomes. The first ADC drug approved by the FDA was gemtuzumab ozogamicin (Mylotarg), which conjugates calicheamicin to an anti-CD33 antibody (Norsworthy et al., 2018). Mylotarg was approved for the treatment of AML in 2000; however, the drug was withdrawn from the US market in 2010 due to validation study failure (Norsworthy et al., 2018). Another ADC drug is Kadcyla, which was approved in 2013 for the treatment of HER-2-positive breast cancer (Chen et al., 2016). Kadcyla is a humanized monoclonal antibody-drug conjugate of ado-trastuzumab emtansine (Kadcyla; T-DM1). Rovalpituzumab tesirine is an ADC drug for the treatment of lung cancer and is currently undergoing clinical trials. Rovalpituzumab tesirine conjugates a humanized anti-delta-like protein-3 (DLL3) antibody to pyrrolobenzodiazepine through a proline-alanine dipeptide, which can be cleaved by proteases and is used to treat SCLC and giant cell neuroendocrine carcinoma (Lashari et al., 2018). However, very few related drugs in clinical trials have been reported for NSCLC therapy. *In vitro* recombinant proteins conjugated to anti-tumor drugs, which are used for specific targeted therapies. Liu et al. constructed several novel fusion proteins, including Ec-LDM-TF, in which EGF ligand oligopeptides specifically recognize receptors that are highly expressed in tumor cells (Liu et al., 2014, 2018a, 2018b). Tuffsin (TF), an endogenous small-molecule polypeptide, which plays an important role in the body's natural immune system (Fridkin and Najjar, 1989), has robust anti-tumor activity (Khan et al., 2007) and is a new therapeutic agent with multiple functions. The comprehensive

analysis of *in vitro* biological activities of drugs were conducted to evaluate their mechanism of action, and the anti-tumor activity by using different mouse models of transplanted tumors were also performed (Liu et al., 2014, 2018a, 2018b). The data indicated that multifunctional drugs have excellent application potential. Furthermore, many studies have found that the cross-talk between EGFR and IGF-1R may be a factor underlying the development of EGFR-targeted drug resistance (Yeo et al., 2015; Peled et al., 2013).

After targeted therapy failure, the tumor stroma under pathological conditions supports cancer cell survival and proliferation and further promotes the development of metastatic disease. A number of drugs targeting matrix components to prevent this resistance are currently under different stages of preclinical and clinical development. Compared with traditional, individual chemotherapy drugs, monoclonal antibodies targeting EGFR, such as cetuximab or the TKIs gefitinib, erlotinib, and afatinib, can improve the survival rate of patients with NSCLC (Rotow and Bivona, 2017). However, patients often develop drug resistance within a few months, which limits the effectiveness of treatment.

Table 5. Selected clinical NSCLC trials evaluating combinations of targeted therapies

Drug regimen	Patients	Phase	Result	Clinicaltrials.gov identifier*
EGFR TKI + chemotherapy				
Erlotinib +	Stage IIIB–IV	III	PFS 7.6 months	NCT00883779

chemotherapy	NSCLC			
Gefitinib + chemotherapy	EGFRm, EGFR-T790M+ prior EGFR TKI	III	PFS 5.4 months	NCT01544179
Gefitinib +Platinum-doublet	EGFRm, EGFR-T790M+ prior EGFR TKI	III	OS 50 months	NEJ009 trial
EGFR-TKI + bypass pathway				
Erlotinib + Cabozantinib; Gefitinib + Capmatinib	EGFRm, prior EGFR TKI, MET-amplified, RET rearrangement, wild-type EGFR	II, I/II	15% PR; ORR 0% for combination arm; PFS 4.7 (combination) versus 1.8 months (erlotinib)	NCT01610336, NCT00596648
Osimertinib+ Navitoclax (BCL-2 family inhibitor)	EGFR-T790M+ prior EGFR TKI	I	ongoing	NCT02520778
Gefitinib + BKM120	EGFR overexpression or PIK3CA	Ib	PFS 2.8 months	NCT01570296

	mutation, prior EGFR TKI			
EGFR TKI + anti-EGFR/VEGF mAbs				
Osimertinib+ Necitumumab/ Ramucirumab	EGFR-T790M+ prior EGFR TKI	I	ongoing	NCT02789345
Erlotinib+ Bevacizumab	EGFRm	III	ongoing	NCT01532089
EGFR TKI + ICI				
Osimertinib+ Durvalumab	prior EGFR TKI	1b	ongoing	NCT021434466
Carboplatin/Paclitaxel + Bevacizumab+ Atezolizumab	EGFRm, ALKm	III	PFS 9.7	NCT01903993, NCT02008227
ALK inhibitor combinations				
Ceritinib +Everolimus (mTOR inhibitor)	ALK+ NSCLC, prior ALK TKI	I/Ib	Ongoing	NCT02321501
Ceritinib +Trametinib (MEK inhibitor)	ALK^+; with or without prior ALK TKI	I/II	Ongoing	NCT03087448
Ceritinib + LEE011	ALK^+ NSCLC,	I/Ib	Ongoing	NCT02292550

(CDK4/6 inhibitor)	prior ALK TKI			
Alectinib +Bevacizumab	ALK^+ NSCLC	II	Ongoing	NCT02521051
Crizotinib +Nivolumab/Ipilimumab or +Pembrolizumab	ALK^+ NSCLC; ALK^+ advanced NSCLC	I/Ib	PFS 24.1, OS 47.2	NCT01998126, NCT02511184
Crizotinib/Lorlatinib +Avelumab	ALK^+ NSCLC	1b	Ongoing	NCT02584634

*More clinical trial details can be found on the clinicaltrials.gov website.

6. Future perspectives

Different types of sequencing analysis of clinical samples revealed the molecular taxonomy of lung cancer and the complexity of somatic alterations in NSCLC, including epigenomic modifications, transcription factors, splicing factors, and genes involved in cellular immunity (Schrank et al., 2018; Rotow and Bivona, 2017). NSCLC is heterogeneous and is characterized by distinct genetic abnormalities within a single tumor and between the primary and metastatic tumor. The genetic complexity of NSCLC is one of the major causes of off-target effects of TKI-targeting drugs. Unfortunately, effective gene-targeting drugs have not yet been developed. In-depth understanding of the mechanisms underlying drug resistance in patients with lung cancer should facilitate the development of new treatment strategies.

At present, the foundation for developing “multifunctional comprehensive treatment” and

"precision treatment" is being established by collecting the comprehensive information from individual lung cancer patient. The future NSCLC treatment program will be genotype-dependent, with a strictly selected combination of drugs, ensuring precise targeting and robust killing of tumor cells, enhancing the tumor immune response, inhibiting angiogenesis, and blocking the interaction between tumor cells and other non-cellular components simultaneously. In NSCLC-targeted therapy, tumors are constantly changing, which may further induce heterogeneity and affect therapeutic outcome. Therefore, it is very necessary to comprehensively analyze the changes in tumor cells and their surrounding environment to gain an in-depth understanding of the molecular mechanisms underlying the development of drug resistance. In addition, the dynamic changes in factors driving tumor growth during treatment should also be monitored for the development of effective, individualized treatment regimens.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (NO.81502691 and NO.81573246); the China Postdoctoral Science Foundation Grant (2017M622188).

We would like to thank the NIH Fellows Editorial Board for providing assistance with language editing and proofreading. We sincerely apologize to the researchers whose studies could not be cited due to space limitations.

Contributions

Jian Xu and Wen-juan Liu wrote and edited the manuscript. Yue Du designed figures.

Ming Yang and Ru Wen contributed to discussion of the content.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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