Am J Cancer Res 2024;14(6):2994-3009
www.ajcr.us /ISSN:2156-6976/ajcr0155506

Review Article

The role of m6A methylation in targeted therapy resistance in lung cancer

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Received January 18, 2024; Accepted May 28, 2024; Epub June 15, 2024; Published June 30, 2024

Abstract: Targeted therapies have greatly improved clinical outcomes for patients with lung cancer (LC), but acquired drug resistance and disease relapse inevitably occur. Increasingly, the role of epigenetic mechanisms in driving acquired drug resistance is appreciated. In particular, N6-methyladenosine (m6A), one of the most prevalent RNA modifications, has several roles regulating RNA stability, splicing, transcription, translation, and destruction. Numerous studies have demonstrated that m6A RNA methylation can modulate the growth and invasion of cancer cells as well as contribute to targeted therapy resistance in LC. In this study, we outline what is known regarding the function of m6A in the acquisition of targeted therapy resistance in LC.

Keywords: m6A methylation, lung cancer, targeted therapy resistance, epidermal growth factor receptor tyrosine kinase inhibitors

Introduction

Among all cancer types, lung cancer (LC) is associated with the highest morbidity and secondary highest mortality rate in the world, and it is therefore an important public health problem [1, 2]. LC comprises non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), which are defined according to histological characteristics. NSCLC represents approximately 85% of LCs and is further divided into lung squamous carcinoma (LUSC), lung adenocarcinoma (LUAD), and other rare types [3], while SCLC accounts for the remaining approximately 15% of LCs. Surgery is the primary treatment for loco-regional LC, but 30-40% of patients are diagnosed with advanced disease due to insidious onset, and curative-intent resection is not suitable for these patients [4]. Radiotherapy and chemotherapy are used in the treatment of both loco-regional and advanced LC, but with limited clinical benefit as well as toxicities that often limit patient adherence [5]. Although no druggable driver mutations have been identified for SCLC, molecular targeted therapies have greatly increased patient survival in NSCLC and other cancer types [6]. In particular, patients with epidermal growth factor receptor (EGFR)-mutant NSCLC may benefit from EGFR-targeted therapy. Several other targeted therapies, gene therapies, and immunotherapies are also now approved to treat certain LC subtypes according to molecular characteristics [7, 8]. Unfortunately, even when there is an initial clinical benefit, resistance to targeted therapy is a major cause of treatment failure in LC, which may lead to tumor progression, distant metastasis, or recurrence [9, 10].

Single-drug and multi-drug resistance both occur in LC, and lack of drug response can be either inherent or acquired after treatment [11]. Enhanced drug efflux, decreased drug influx, cancer stem cell properties, autophagy levels, and other mechanisms can all contribute to the resistance of cancer cells to targeted therapy (**Figure 1**).

In addition, advancements in the fields of DNA methylation, histone modification, chromatin remodeling, and RNA modification have con-

https://doi.org/10.62347/LX0S2662

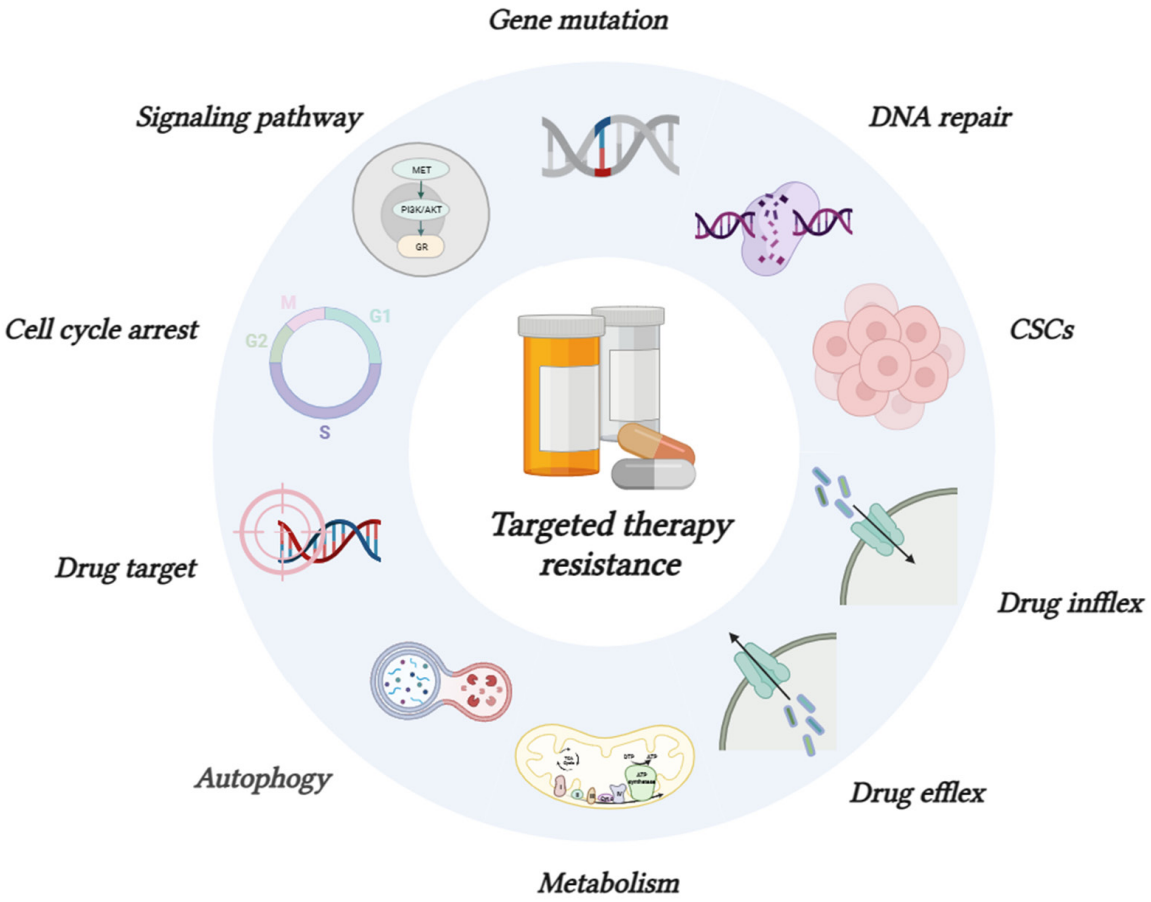

Figure 1. The mechanism of targeted drug resistance. Gene mutation and DNA damage repair; Generation of cancer stem cells; Reduced drug influx and increased drug efflux; Alterations in signal pathways and metabolism; Induction of autophagy; Secondary drug target mutation; Cell cycle arrest.

firmed that treatment resistance in LC is significantly influenced by epigenetics [12]. Historically, technical issues have hindered progress in RNA modification research, which dates back to the 1970s; however, a novel method that combines RNA immunoprecipitation with next-generation sequencing, which was developed in 2012, has helped the field advance [13]. Currently, over 170 different kinds of RNA chemical modifications have been identified. More than 50% of RNA alterations involve RNA methylation, including N1-adenylate methylation (m1A) and generation of N6-methyladenosine (m6A), 5-methylcytosine (m5C), and N7-methylguanine (m7G) [14, 15]. In eukaryotic cells, internal mRNA modifications such as m6A and m1A often foster mRNA stability, and m6A is a prevalent modification known to profoundly affect mRNA splicing, transport, translation, and other activities [16]. Meanwhile, modifications to the 5' cap and 3' poly(A) tail play a critical role in the regulation of transcription [17].

The dynamic, reversible process of m6A methylation is controlled by so-called "writers", "readers", and "erasers" and, together, these enzymes regulate key biological processes [18-21]. Among methyltransferases, the methyltransferase-like 3 (METTL3), methyltransferase-like 14 (METTL14), methyltransferase-like protein 7B (METTL7B), Wilms tumor 1-associating protein (WTAP), zinc finger CCCH-type containing 13 (ZC3H13), and KIAA1429 (VIRMA) are known to act as m6A writers. A core component of METTL3 exhibits catalytic activity, and METTL14 plays a role in substrate recognition. WTAP is in charge of enlisting METTL3 and METTL14 and fusing them with other elements to create hybrids. So far, only two m6A erasers have been identified: the demethylases fat

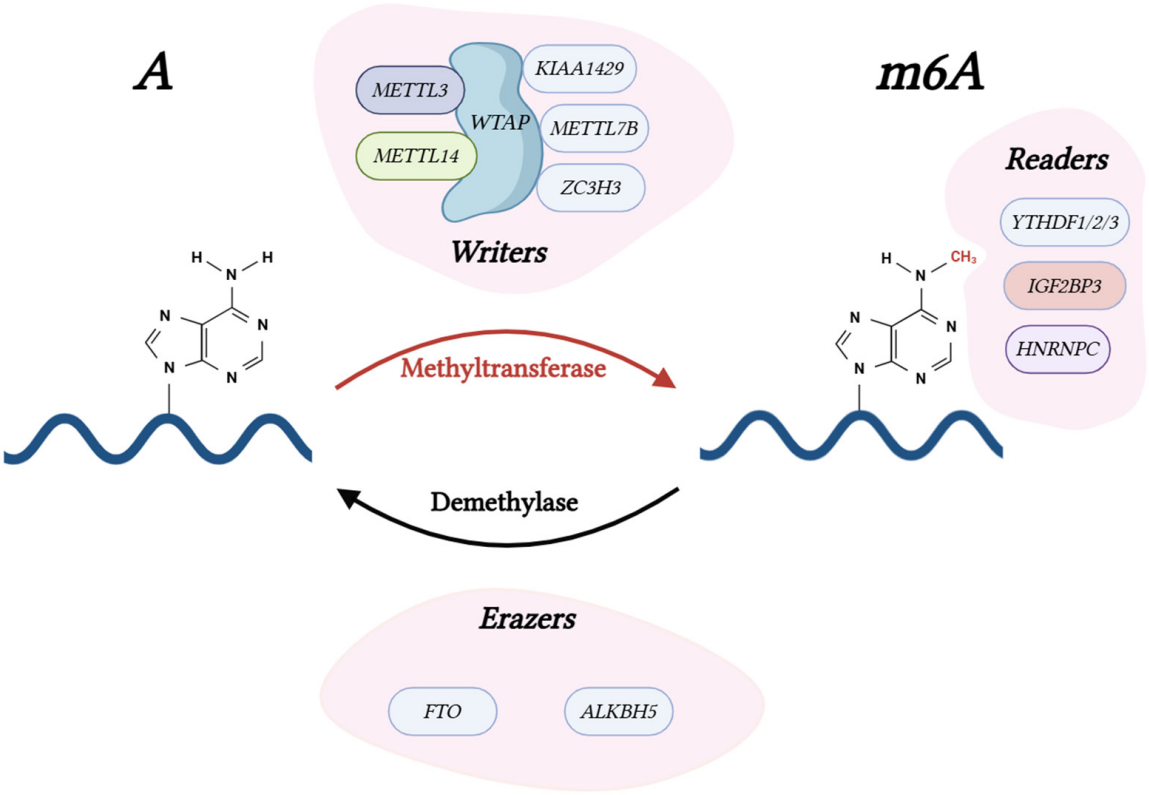

Figure 2. The m6A methylation process. RNA gets methyl groups added by the "writers". The function of METTL3 and METTL14 is substrate recognition. The hiring of METTL3, METTL14, and other components is the responsibility of WTAP. The "readers" recognize m6A and impact different RNA functions; they mostly include members of the YTH domain-containing family (YTHDF1/2/3) that have the ability to degrade mRNA. The "erasers" (FTO and ALKBH5) delete the m6A modification.

mass and obesity-associated gene (FTO) and AlkB homolog 5 (ALKBH5). FTO, the first identified RNA demethylase, is tightly linked to obesity and cancer and shares a core domain structure with ALKBH5. Readers are the proteins that identify methylation sites and use the information to engage biological pathways. Readers include YT521-B homology (YTH) m6A RNA-binding protein 1 (YTHDF1), YTH m6A RNA-binding protein 2 (YTHDF2), YTH m6A RNA-binding protein 3 (YTHDF3), insulin-like growth factor-2 mRNA-binding protein 3 (IGF2-BP3), and heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2B1). Among these, proteins with the YTH domain selectively bind to methylated mRNA and regulate translation and degradation farther along the translational pathway (**Figure 2**). Genetic mutations and environmental factors are the usual causes of the dysregulation of m6A modulators, modifiers, and mediators in cancer, and various underlying mechanisms have been proposed.

Growing evidence points to the critical role of m6A methylation in the regulation of gene expression. However, the function of m6A in resistance of LC to targeted therapies has only recently come to the forefront. Here, we review the current state of knowledge regarding to role of m6A RNA methylation in therapy resistance in LC as well as potential strategies overcome it.

Targeted therapy resistance and m6A

Tumor profiling has enabled the selection of personalized targeted therapies to treat some patients with LC. Currently approved targeted therapeutic agents for NSCLC inhibit driver genes, such as EGFR, anaplastic lymphoma kinase (ALK), c-ROS oncogene-1 (ROS1), v-RAF murine sarcoma viral oncogene homolog B (BRAF), mesenchymal-epithelial transition (MET), rearranged during transfection (RET), and neurotrophic tropomyosin receptor kinase

(NTRK) [22]. In particular, tyrosine kinase inhibitors (TKIs) targeting EGFR have shown significant efficacy in the treatment of LC. It has also been shown that m6A modifications may be involved in the development of alterations in driver genes associated with drug sensitivity and poor prognosis in LC. There are no currently no targeted therapies approved to treat SLSC due to the rarity of driver mutations in these tumors.

Function of m6A in LC

m6A alteration of numerous tumor-related genes in LC is indicative that m6A plays a functional role in this context. Numerous investigations have shown that regulation of oncogenes and tumor suppressor genes by m6A is essential for the genesis, development, and metastasis of LC, and these mechanisms were summarized by Zhang et al. in their review study [23]. m6A is a tumor driver that inhibits the expression of tumor suppressor genes while promoting the production of oncogenes, and it contributes significantly to the development of LC. Here, we go into further detail to describe current knowledge regarding the function of m6A in lung cancer.

METTL3 promotes the malignancy of NSCLC by inhibiting secreted frizzled-related protein (SFRP2) stability through m6A modification, which activates the Wnt/β-catenin signaling pathway [24]. Additionally, Chen et al. proved that METTL3-mediated upregulation of Rac family small GTPase 3 (RAC3) activated the protein kinase B (AKT)/nuclear factor-kappa B (NF-κB) pathway, which was responsible for cancer-associated fibroblasts' promoting effect on NSCLC cell migration [25]. One possible therapeutic target for the treatment of NSCLC may be METTL3. Methyltransferase-like 16 (METTL16) is an additional m6A methyltransferase. Wang et al. identified eukaryotic translation initiation factor 4E family member 2 (eIF4E2), an interactor of METTL16, which promotes lung tumorigenesis by acting as a competitor of eIF4E to inhibit translation [26]. Li et al. discovered that HNRNPA2B1's m6A target lncRNA MEG3 was inhibited, which resulted in a drop in MEG3's m6A levels but an increase in its mRNA levels. Moreover, lncRNA MEG3 may function as a miR-21-5p sponge, upregulating phosphatase and tensin (PTEN) and deactivating phosphatidylinositol 3-kinase (PI3K)/AKT signaling to inhibit cell invasion and proliferation [27]. Yang et al. showed that the m6A reader IGF2BP3 induces LUAD partial epithelial-mesenchymal transition and metastasis through minichromosome maintenance complex component 5 (MCM5)/Notch axis [28]. Zhang et al. found that methylation of the 5' untranslated region (UTR) internal ribosome entry site (IRES) of vascular endothelial growth factor A (VEGFA) mRNA boosts cap-independent translation and encourages angiogenesis to support the development of lung tumor by recruiting the YT521-B homology domain containing 2 (YTHDC2)/eukaryotic translation initiation factor 4G (eIF4GI) complex [29]. Gao et al. found that FTO activates the focal adhesion kinase (FAK) signaling pathway by upregulating fibroblast activation protein (FAP), which promoted NSCLC metastasis. In addition, defactinib (VS6063), a specific FAK inhibitor, inhibited the pro-metastatic effects of FTO, suggesting a new approach to treating NSCLC metastasis [30, 31].

The fact that m6A modification is associated with the occurrence, progression, and metastasis of lung cancer suggests that m6A modification regulators have great clinical potential and offer considerable prospects and directions for the treatment of lung cancer.

EGFR signaling in LC

The EGFR pathway is crucial in the initiation and progression of NSCLC. EGFR, a transmembrane tyrosine kinase (TK) receptor, triggers a cascade of signaling events that promotes cell proliferation, survival, migration, and differentiation upon binding to the epidermal growth factor (EGF) or other ligands. However, abnormal EGFR pathway activation can result in uncontrolled cell proliferation and tumor formation [32].

In LC, multiple mechanisms can lead to abnormal activation of the EGFR pathway. Mutations in the EGFR gene, particularly exon 19 deletion and L858R mutation in exon 21, represent the most common activation mechanisms [33, 34]. Even in the absence of EGFR mutations, alterations or overactivation of downstream signaling molecules, such as rat sarcoma (Ras), rapidly accelerated fibrosarcoma (Raf), mitogen-activated protein-kinase kinase (MEK), and extra-

cellular regulated protein kinases (ERK), can cause abnormal EGFR pathway activation [35]. Additionally, amplification or activation of other signaling pathways, such as MET and Human Epidermal GrowthFactor Receptor 2 (HER2), can directly activate downstream signaling pathways, bypassing EGFR and promoting tumor cell growth and survival. An increase in the copy number of EGFR can also lead to its overexpression and enhanced activity to promote tumor cell growth and survival [36].

Abnormal EGFR pathway activation leads to various biological effects, including enhanced cell cycle progression, inhibition of apoptosis, promotion of angiogenesis, and tumor metastasis, all of which significantly contribute to LC development and progression. Consequently, therapeutic targeting of the EGFR pathway has emerged as an important strategy in treating NSCLC [37]. However, resistance to anti-EGFR therapies poses a substantial clinical challenge [38].

Three generations of EGFR-TKIs

TK activation by competitive binding of endogenous ligands to EGFR is inhibited by epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs), which are small molecules that block the EGFR signaling pathway and inhibit the proliferation and metastasis of tumor cells. First-generation EGFR-TKIs included gefitinib, erlotinib, and icotinib; subsequently, the second-generation EGFR-TKIs afatinib and dacomitinib and third-generation osimertinib were developed [39]. EGFR-TKIs are now established first-line treatment options for advanced EGFR-mutated NSCLC. In-frame deletions in exon 19 and single-point mutations in exon 21 (L858R) are the most prevalent activating mutations and are approved as companion diagnostics for the selection of EGFR-TKI therapy in NSCLC [33, 40]. In molecularly selected patients, first- and second-generation EGFR-TKI therapy extended progression-free survival (PFS) as compared to conventional chemotherapy [41, 42]. However, acquired T790M was occurs in about 50% of patients receiving first-generation or second-generation EGFR-TKIs [43, 44] and results in treatment resistance. Osimertinib is effective in some of these patients who developed resistance to earlier EGFR-TKI therapies. Currently, osimertinib is the first-line treatment of choice for patients with EGFR-mutated NSCLC after the FLAURA trial (NCT02296125) demonstrated a significantly improved median PFS of 18.9 months in the Osimertinib group, which was much longer than the PFS achieved with first-generation EGFR-TKIs [45]. Unfortunately, even with objective response rates in the treatment-naive condition as high as 70-80%, EGFR-TKI resistance invariably develops [33, 40].

The role of m6A in EGFR-TKI resistance

An increasing body of research indicates that m6A methylation may be a useful biomarker for cancer cells resistant to treatment [46-48]. In fact, recent studies have found that numerous m6A-modifying enzymes are involved in EGFR-TKI drug resistance, suggesting that m6A-modifying enzymes may be novel drug targets to achieve durable clinical responses in NSCLC.

EGFR-TKI resistance and m6A writers: Numerous studies have demonstrated the strong correlation between treatment sensitivity and m6A writers. The m6A writer METTL3 is currently the most intensively studied, and METTL3 is overexpressed in NSCLC. Zhang et al. suggested that the lncRNA SNHG17/Enhancer Of Zeste Homolog 2 (EZH2)/large tumor suppressor kinase 2 (LATS2) axis could be a potential therapeutic target for gefitinib resistance in patients with LUAD, as it promotes gefitinib resistance through epigenetic repression of LATS2 and recruitment of EZH2 to LATS2 [49]. Based on the findings of Jia et al., both in vivo and in vitro gefitinib resistance can be regulated by LINC00969. LINC00969 complexes with EZH2 transcriptionally controls the amount of histone H3 lysine 27 methylation (H3K27me3) in the pyrin domain containing 3 (NLRP3) promoter region, and LINC00969 complexes with METTL3 post-transcriptionally modifies the m6A level in Nod-like receptor, NLRP3 in a m6A-YTHDF2-dependent manner. With more m6A modification of NLRP3, NLRP3 expression is epigenetically suppressed, which inhibits the activation of the NLRP3/caspase-1/gasdermin D (GSDMD)-related classical pyroptosis signaling pathway, conferring an anti-pyroptotic phenotype and driving gefitinib resistance in LC [50]. According to Gao et al., METTL3 also alters MET levels to control the phosphatidylinositol 3-kinase (PI3K)/AKT signaling, which

results in gefitinib resistance in LUAD [51]. Another study found that METTL3 enhances the stability of mRNAs involved in autophagy, including autophagy-related 5 (ATG5), autophagy-related 7 (ATG7), microtubule-associated protein 1 light chain 3 (LC3B), and sequestosome-1 (SQSTM1), leading to gefitinib resistance [52].

Along with the above-mentioned pyroptosis, autophagy, apoptosis, and cancer stem cell (CSC) stemness are also involved in therapy resistance. The proliferation and survival of CSCs are associated with the evolutionarily conserved Notch signaling pathway [53]. Overexpression of the lncRNA tumor suppressor candidate 7 (TUSC7) can prevent tumor cells from proliferating and invading [54], and Li et al. discovered that erlotinib-resistant LUAD cells exhibit TUSC7 suppression and Notch signaling activation. They further demonstrated that YTHDF2 blocked TUSC7 expression and that METTL3 continuously stimulated miR-146a/Notch signaling. In LUAD cells, both effects aided in the development of drug resistance [55].

One study suggested that combination treatment with β-elemene - the primary active component of the anti-cancer medication elemene, derived from the Chinese medicinal plant Curcuma Wenyujin [56] - and erlotinib could enhance sensitivity to erlotinib through induction of ferroptosis via increased lncRNA H19 expression in LC with primary erlotinib resistance [57]. In fact, treatment with β-elemene can also reduce METTL3-mediated autophagy in NSCLC and, hence, reverse cellular resistance to gefitinib [52]. Together, the use of TKIs and inhibitory lncRNAs specific to Notch or natural products from traditional medicine may be novel effective therapeutic strategies for LC.

The relationship between osimertinib and m6A readers has been demonstrated to involve the Notch signaling pathway. The well-known type 2 diabetes medication metformin markedly slows tumor growth in the fasted state [58]. METTL3 attenuates the effect of micro RNA (miRNA) Let-7b on Notch-signaling-related stem cell renewal by increasing pri-Let-7b and decreasing mature and pre-Let-7b. Metformin inhibits DNA methyltransferase-3a/b (DNMT3a/b) binding to the METTL3 promoter with the help of m6A readers NF-kappaB activating protein (NKAP) and HNRNPA2B1, and metformin-stimulated Let-7b maturation improves response to osimertinib therapy [59]. However, a randomized, double-blind phase II trial showed no significant advantage of metformin in combination with gefitinib in terms of either PFS or overall survival (OS) and, therefore, the trial did not support its concomitant use in patients with NSCLC without a history of diabetes when treated with first-line EGFR-TKIs [60].

Cancer cells have unique metabolic characteristics and undergo considerable metabolic remodeling. In addition, m6A writers have been shown to affect redox homeostasis in the tumor microenvironment (TME), which contributes to therapy resistance. Increased METTL7B in LUAD cells upregulated the protein levels and enzymatic activity of three antioxidant enzymes - glutathione peroxidase 4 (GPX4), superoxide dismutase1 (SOD1), and haem oxygenase 1 (HMOX1) - through m6A alteration, thereby increasing reactive oxygen species (ROS) scavenging in the TME and contributing to TKI resistance. Increased m6A levels induced by METTL7B overexpression resulted in gefitinib and osimertinib resistance in LUAD, and this resistance was dependent on ROS scavenging [61]. These findings support the use of METTL7B inhibitors in conjunction with TKIs in patients who are resistant to EGFR-independent TKIs.

Recent studies implicated another methyltransferase, KIAA1429, in the progression and therapeutic resistance of cancer [62]. Tang et al. found that KIAA1429 was increased in gefitinib-resistant NSCLC cells, and that high KIAA1429 expression was indicative of a poor prognosis. By improving homeobox A1 (HOXA1) mRNA stability, KIAA1429 enhances gefitinib resistance in NSCLC cells [63]. According to Lin et al., KIAA1429 expression was higher in LUAD tissues than in normal adjacent lung tissues. This increased expression of KIAA1429 additionally contributed to gefitinib resistance and metastasis in NSCLC cells. Mechanistically, studies showed that the c-Jun amino-terminal kinases (JNK)/mitogen-activated protein (MAPK) pathway is activated and gefitinib resistance is induced as a result of KIAA1429 promoting m6A modification of mitogen-activated protein kinase kinase kinase 2 (MAP3K2) [64]. Overall, these results convincingly nominate

KIAA1429 as a novel druggable target in gefitinib-resistant NSCLC.

EGFR-TKI resistance and erasers: Dysregulation of m6A demethylases is also commonly observed in treatment-resistant LC. Our understanding of epitranscriptomic regulation mechanisms has improved owing to the discovery of the m6A demethylase known as the FTO [65]. Breast cancer resistance protein (BCRP) and multidrug resistance protein-7 (MRP7) play a key role in gefitinib resistance, and the ATP-binding cassette (ABC) transporters family was revealed to mediate gefitinib treatment sensitivity [31, 66, 67]. The ABC family of proteins is primarily responsible for TKI efflux. According to Xiao et al., ATP binding cassette subfamily C member 10 (ABCC10) levels were increased in an m6A-dependent manner, and exosomal transfer of FTO could contribute significantly to exosome-mediated transmission of gefitinib resistance. Both FTO knockdown and FTO decrease in donor exosomes improved gefitinib sensitivity in gefitinib-resistant cells, and recipient gefitinib-resistant PC9 cells also acquired resistance to gefitinib [68]. Chen et al. discovered that gefitinib and meclofenamic acid (MA), a highly selective FTO inhibitor and non-steroidal anti-inflammatory medication, had synergistic effects on inducing apoptosis NSCLC cells. As a result of the FTO/M6A/MYC axis-mediated downregulation of BCRP and MRP7, their findings imply that MA functions as a an agent that can sensitize resistant NSCLC cells to gefitinib [69]. Additionally, BCRP and MRP7 are two efflux pumps known to contribute to gefitinib resistance, and gefitinib was shown to be a substrate of BCRP and MRP7 [67], making these two efflux pumps prospective targets for overcoming gefitinib resistance in NSCLC. Serum exosomal m6A demethylase FTO enhances gefitinib resistance in patients with NSCLC by upregulating the expression of its downstream genes fibronectin leucine-rich transmembrane protein-3 (FLRT3), prostacyclin (PGI(2)) synthase (PTGIS), and signal regulatory protein (SIRP). The prognosis risk assessment model based on the three downstream genes of FTO could predict the prognosis of patients with NSCLC [70]. Taken together, current research suggests that targeting FTO-mediated gefitinib resistance may represent a novel strategy to improve clinical outcomes for patients with NSCLC.

EGFR-TKI resistance and readers: Multiple studies have shown that m6A readers may be involved in regulating EGFR-TKI resistance by modulating gene expression and translation as well as modulating apoptotic signaling pathways. The first confirmed m6A readers were members of the YTH family of proteins. YTHDF2 can bind with hundreds of different target RNAs through m6A modifications in its capacity as an m6A reader [71]. Further endoribonucleolytic cleavage of circular RNAs (circRNAs) is mediated by YTHDF2-HRSP12-RNase P/MRP in a YTHDF2-dependent manner [71]. Significant enrichment of m6A-modified circASK1 was discovered by Wang et al. in gefitinib-resistant cells, and this enrichment may have been mediated by METTL3. The ASK1/JNK/p38 signaling pathway is closely related to apoptosis [72, 73], and m6A-modified circASK1 is recognized by YTHDF2, which promotes circASK1 decay and downregulation. circASK1 encodes the ASK1 isoform ASK1-272a.a, which competes with full-length ASK1 for binding with Akt. This, in turn downregulates Akt-mediated S83 phosphorylation. The end result is rescuing of the proapoptotic activity of ASK1/JNK/p38 signaling, thereby increasing gefitinib sensitivity by inducing apoptosis in LUAD cells [74].

Regulation of regeneration of stem cells and resistance to therapy by Let-7 family micro RNAs (miRNAs) is associated not only with m6A writers [59] but also with m6A readers. Another investigation concerning osimertinib resistance (OR) found that the m6A reader YTHDF3 facilitated the establishment of a circ-FBXW7/Wnt/Let-7 feedback axis. The capacity of resistant H1975OR cells and HCC827OR cells to self-renew can be inhibited by YTHDF3, by imposing circ-FBXW7, or by both [75]. However, Ji et al. discovered that LUAD's susceptibility to osimertinib was increased by circKRT17 silencing. Through the recruitment of eukaryotic initiation factor 4A-3 (EIF4A3), METTL3 promoted yes-associated protein 1 (YAP1) nuclear localization and, in turn, improved the stability of circKRT17 [76].

Drug resistance and tumor genesis are influenced by intra-tumoral redox homeostasis [77, 78]. It has been convincingly demonstrated that ROS influences EGFR-TKI resistance in LC [79, 80], and Lin et al. discovered that LC resistance to gefitinib and osimertinib involves met-

abolic reprogramming. IGF2BP3 increased cytochrome c oxidase subunit 6B2 (COX6B2) mRNA stability by binding to the 3' UTR untranslated region of the gene in an m6A-dependent way. In lung cancer cells, overexpression of the RNA binding protein IGF2BP3 generally decreased sensitivity to TKIs treatment and accelerated the emergence of TKIs resistance by promoting oxidative phosphorylation [81].

Together, the collective effects of m6A readers on CSC stemness, metabolic reprogramming, apoptosis inhibition may optimize LC-targeted therapy.

The role of m6A in other targeted therapies resistance

Drug resistance in LC has been linked to m6A modifiers, underscoring the possibility of m6A-related molecules as predictive biomarkers. MET is overexpressed in over 40% of LCs, and 4-6% of LUADs harbor ALK mutations [81, 82]. In NSCLC with high expression of c-MET, chidamide, a histone deacetylase inhibitor, downregulates WTAP and METTL3 to induce hypomethylation of c-MET, which lowers c-MET expression and increases sensitivity to the ALK/ROS1/c-MET kinase inhibitor crizotinib [82]. Therefore, co-therapy with chidamide and crizotinib could be a potentially effective new approach for NSCLC with high levels of c-MET expression or c-MET gene amplification.

Further research is underway to find potential m6A modulators among currently available medications, including those that have not been reported to regulate methyltransferases. Chidamide and β-elemene attenuate therapy resistance through their functions as potential inhibitors of m6A methyltransferases [21].

To investigate the functional role of m6A in NSCLC, Meng et al. selected afatinib-resistant and afatinib-sensitive NSCLC cell lines based on characterization in the Genomics of Drug Sensitivity in Cancer (GDSC) and identified functionally m6A-modified genes by difference analysis. They identified that aurora kinase B (AURKB), cyclin-Dependent Kinase 2 (CDK2), ABCC10, etc. were modified in afatinib-resistant but not in afatinib-sensitive cell lines, suggesting that m6A-mediated regulation of the cell cycle may affect treatment response in NSCLC [83]. In another study, Yan et al. developed a novel algorithm based on miRNAs based on m6A-related immune lncRNAs (mrilncRNAs) and determined that, when used with other algorithms based on stage and risk score, it was possible to predict survival outcomes. Moreover, with this approach, patients with LUAD could be stratified into high- and low-risk groups for prognostic signature, which can aid in therapy selection [84]. Shen et al. discovered differential expression of 23 m6A RNA regulators in LUAD and nearby normal tissues. These data were used to develop a lncRNA score model, and they demonstrated that the lncRNA score could predict clinical response to immunotherapy [85]. The four-differentially expressed m6A-related gene signature (ZC3H13, Cbl proto-oncogene like 1 (CBLL1), ELAV like RNA binding protein 1 (ELAVL1), and YTHDF1) was validated to be significantly associated with clinical characteristics by univariate and multivariate Cox analysis in a study of 1,039 patients with LC from The Cancer Genome Atlas (TCGA) dataset, which suggested its potential use in forecasting the prognosis of LC [86].

Mechanisms through which drugs can alter m6A methylation

Why do targeted therapies change m6A methylation status? There could be two causes for this. On the one hand, drugs affect methylation levels because they directly control the expression of specific m6A genes. For example, osimertinib upregulated the expression of METTL7B, while gefitinib upregulated the expressions of METTL3. These methyltransferase changes increased the m6A methylation in vivo [61]. On the other hand, therapies can affect methylation levels by affecting the activity of m6A readers, writers, and erasers. For example, Dai et al. found that gifitinib increased LINC00969 expression and activated m6A modification in LC cells. LINC00969 promotes m6a modification of NLRP3 by binding to METTL3. This promotes NLRP3 degradation to suppress the pyroptosis signaling pathway, thereby increasing TKI resistance in LC [50].

Some scholars categorize m6A-related gene products into three groups using the Feinberg et al. cancer epigenetics classification scheme [87]: enzymes that directly add or remove m6A marks from RNAs or reader proteins that decipher the marks are known as m6A modifiers

(or regulators); m6A modulators are the upstream regulators of m6A modifiers; and m6A mediators, which are tumor suppressors or downstream oncogenes controlled by m6A modifiers. Genetic mutations and environmental factors are the usual causes of the dysregulation of m6A modulators, modifiers, and mediators in cancer, and various underlying mechanisms have been proposed.

First, through control of m6A modifiers, a genetic mutation of m6A modulators, which are typically driver genes in cancer, can result in reprogramming of the m6A epitranscriptome. Second, the activity of m6A modifiers can change due to genetic modifications. Third, loss of m6A motif sequences or increase of de novo m6A sites are two possible outcomes of gene mutations altering m6A mediators. In fact, over 5,000 disease-associated genetic variants that disrupt m6A-target DRACH motifs have been linked to pathogenesis of many diseases by a comprehensive epitranscriptomic investigation [88]. Fourth, changes in m6A modulators can have a solo or combined impact on m6A signaling by influencing the expression and/or activity of m6A modifiers. Modified transcriptional regulation by transcription factors, DNA, and/or histone changes; post-transcriptional regulation by non-coding RNAs; and post-translational regulation through protein modifications are some of these effects. Fifth, to change the m6A epitranscriptome in cancer cells, m6A modulators also transduce signals brought on by external stimuli and ligands of cellular receptors. Finally, the m6A methylation and demethylation processes can be directly impacted by changes in the level of epigenetic metabolites. Notably, through a variety of feedback loops, m6A modification itself typically has an impact on m6A modulators and, consequently, m6A modifiers [89].

Methods of determining the m6A methylation

Techniques such as liquid chromatography-tandem mass spectrometry and RNA immunoblotting can be used to easily and accurately detect global m6A levels. In addition, next-generation sequencing's transcriptome-wide profiling of m6A can determine locus-specific alterations in m6A modification, which has expanded knowledge of the diverse functions that m6A plays in post-transcriptional regulation of gene expression. Several high-throughput m6A-specific sequencing methods are available, each with unique advantages and disadvantages.

Methylated RNA immunoprecipitation sequencing (MeRIP-seq) efficiently and affordably determines human m6A methylation levels in a high-throughput manner. Immunomagnetic beads conjugated to m6A antibodies can be used to enrich m6A-methylated mRNA fragments for subsequent MeRIP sequencing analysis [87]. This approach requires ≥300 μg of total RNA as the starting material and yields m6A maps with a resolution of around 100-200 nucleotides. More recently, several improved antibody-based techniques have been developed, offering the advantages of greater resolution, decreased requirement for starting material, and/or the ability to detect m6A quantitatively. m6A individual nucleotide resolution cross-linking and immunoprecipitation (MiCLIP) or m6A- cross-linking immunoprecipitation (CLIP) [90, 91], m6A-level and isoform-characterization sequencing (m6A-LAIC-seq) [92], m6A-Crosslinking-Exonuclease-sequencing (m6ACE-seq) [93], m6A-seq [94], and super-low input m6A-seq (SLIM-seq) [95] are a few examples. However, antibody-based approaches continue to fall short of providing stoichiometric m6A data at single-base resolution, which is critical for functional analyses. As a result, numerous antibody-independent m6A-profiling techniques, such as MazF RNase to cleave RNA (MAZTERseq) [96], m6A-sensitive RNA-endoribonuclease-facilitated sequencing (m6A-REF-seq) [97], m6A selective chemical labeling (m6A-SEAL) [98], m6A-label-seq [99], and Diversity Arrays Technology sequencing (DART-seq) [100], have been created. Each of these methods has certain benefits, such as the capacity to profile m6A with minimal amounts of input RNA and a reduced number of false-positive results. These approaches also have limitations, such as poor resolution, limited applicability to in vitro-grown cells, and inaccurate quantitation of m6A.

Furthermore, m6A-selective allyl chemical labeling and sequencing (m6A-SAC-seq), GLObal Navigation Satellite System Reflectometry Instrument (GLORI), and enhanced tagged-amplicon sequencing (eTAM-seq) - three innovative antibody-independent methods created in the last 2 years - offer quantitative transcrip-

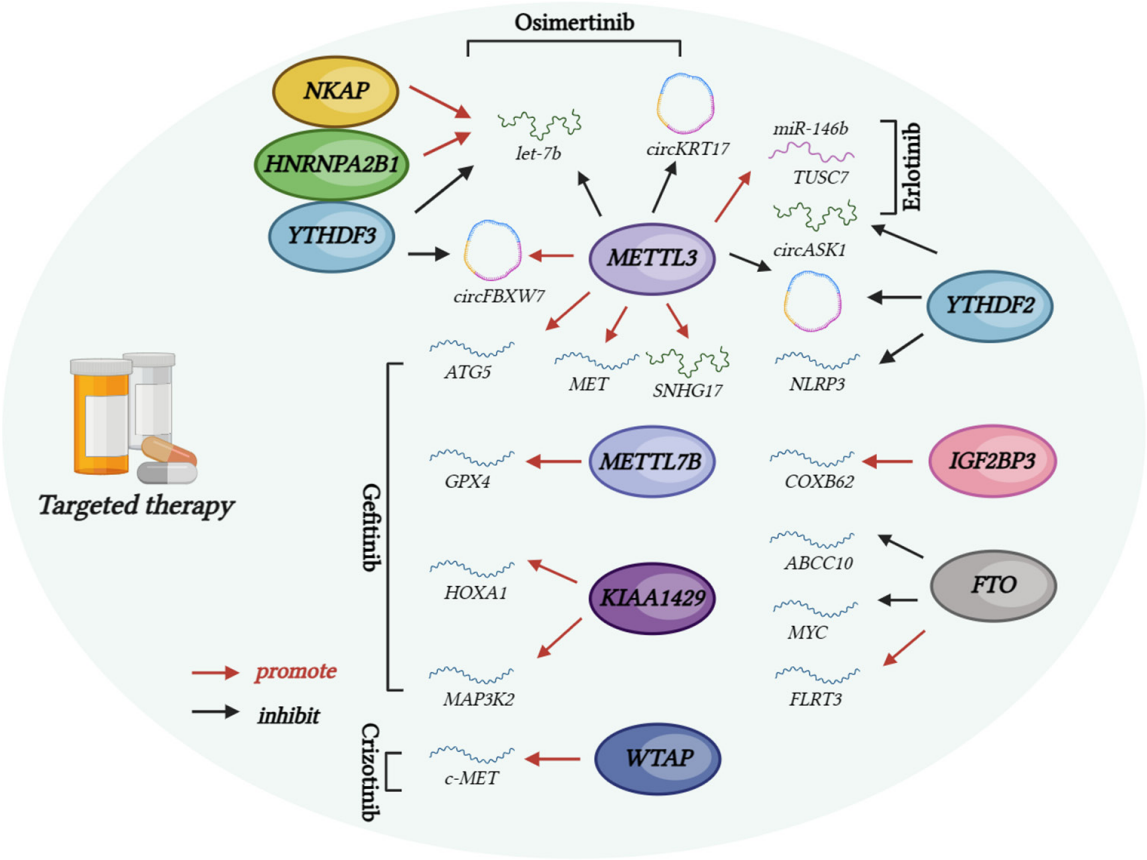

Figure 3. The role of m6A methylation in lung cancer targeted therapy resistance. The different roles that m6A regulators play in lung cancer targeted drugs resistance. "Promote" is indicated by red arrows, and "inhibit" is indicated by black arrows.

tome-wide mapping of m6A with single-base resolution [101-103]. To create mutations during reverse transcription, m6A-SAC-seq takes advantage of Methanocaldococcus jannaschii (MjDim1), an archaeal enzyme that changes m6A into N6-allyl,N6-methyladenosine (a6-m6A). The relative frequencies and locations of these mutations may then be ascertained via RNA-seq. Quantitative m6A maps can be generated using m6A SAC-seq with as few as approximately 30 ng of poly(A)$^+$ RNA molelcules; however, it is less sensitive to Am6AC sequences among DRACH consensus locations than Gm6AC sequences. Although conceptually comparable to bisulfite sequencing of 5-methylcytosine in DNA, GLORI and eTAM-seq differ from m6A-SAC-seq in that they identify unmethylated A by converting A to inosine (I), either chemically (GLORI) or enzymatically (eTAM-seq) [102, 103]. For high-throughput sequencing, eTAM-seq needs a modest amount of starting material (50 ng of poly(A)$^+$ RNA), and, for site-specific m6A quantification, it requires ultra-low levels (250 pg of total RNA). Both approaches determine m6A stoichiometry by subtracting unmethylated A from total A [102, 103]. However, given the significantly larger abundance of A than m6A in RNA (>200-fold), a technique that incorporates direct detection of m6A instead of A is still favored. GLORI and eTAM-seq are unable to distinguish m6A from other adenosine modifications. Third-generation sequencing, or direct sequencing of native RNA usng nanopore technology, is sensitive to RNA modifications and may one day provide a novel technique for detecting such alterations, including m6A [104].

These remarkable technological advances have made it possible to identify m6A quantitatively and precisely with single-nucleotide resolution, increasing our capacity to assess the distributions and impacts of m6A in different cancer and other cell types. The newly devel-

Table 1. The functions of m6A regulators in targeted therapy resistance

Drug	Regulator	Regulaton	Target/Approach	Reference
Erlotinib	METTL3	PR	miR-146a/Notch	[54]
	YTHDF2	PR	TUSC7	[54]
Gefitinib	METTL3	PR	ATG5, ATG7, LC3B, and SQSTM1	[51]
	METTL3	PR	PI3K/AKT	[51]
	METTL3	PR	SNHG17/EZH2/LATS2	[49]
	METTL3	PR	circASK1	[74]
	METTL3	PR	LINC00969	[50]
	METTL7B	PR	GPX4, SOD1 and HMOX1	[61]
	KIAA1429	PR	HOXA1	[63]
		PR	MAP3K2	[64]
	YTHDF2	PR	ASK1/JNK/p38	[72]
	YTHDF2	PR	NLRP3	[50]
	IGF2BP3	PR	COX6B2	[81]
	FTO	PR	ABCC10	[68]
		PR	BCRP and MRP7	[69]
		PR	FLRT3, PTGIS and SIRPα	[70]
Osimertinib	METTL3	PR	Let-7b/Notch	[59]
	METTL3	PR	circKRT17	[76]
	METTL7B	PR	GPX4, SOD1 and HMOX1	[61]
	YTHDF3	PR	circFBXW7/Wnt/Let-7b	[75]
Crizotinib	METTL3	PR	ALK/ROS1/c-MET	[82]
	WTAP	PR	ALK/ROS1/c-MET	[82]

PR: promote resistance.

oped single-nucleotide quantitative methods (m6A-SAC-seq, GLORI, and eTAM-seq) can provide accurate, consistent, and high-resolution maps of m6A methylation at the transcriptome-wide level [101, 103]. Notably, the m6A signals detected in transcripts are largely consistent across different sequencing methods [103]. However, more sophisticated techniques are needed to provide transcriptome-wide quantitative m6A detection at single-base and single-cell resolutions.

Discussion and conclusion

The cellular m6A machinery clearly has essential roles in LC-targeted therapy resistance. Moreover, many oncogenic m6A modifiers have been identified as promising targets for LC therapy. By changing the stability of transcription products from several important genes and activating or inhibiting specific signaling pathways, m6A methylation influences the development of LC-targeted therapy resistance in numerous ways (**Figure 3** and **Table 1**). m6A regulators are good candidates for therapeutic intervention for two main reasons. First, targeting m6A regulators is probably safe because their expression is upregulated in LC but limited in non-malignant tissues and cells in patients. However, because many m6A regulators play critical roles in the resistance of LC cells to cancer therapies, blocking their activity may work in concert with other treatments to improve patient outcomes. However, to date, no inhibitors that target m6A regulators have been tested in the clinical setting.

Given that m6A modifiers typically contribute to targeted therapy resistance through distinct pathways, targeting writers and erasers together is likely achievable and may even have synergistic effects. Functional investigations in animals with genetic depletion of these regulators should validate such synergy between small-molecule inhibitors; conversely, combinatorial genetic deletion could also be used to screen for synergy. Because of the remarkable ability of tumors to escape targeted therapies, medicines targeting m6A regulators may need to be coupled with other therapies to reverse or overcome drug resistance. In sum, comprehending the fundamental function of m6A alteration in LC therapy resistance may yield novel concepts for drug or combination therapy development.

Acknowledgements

Thanks for Dr. Jie Yang's all encouragement and support. This work was supported in part by the National Natural Science Foundation of China (82374273 to Xiaohong Kang and 82074268 to Fei Cao).

Disclosure of conflict of interest

None.

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