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Review Article

The E3 ubiquitin ligase NEDD4 mediates EGFR-TKI acquired resistance in non-small cell lung cancer

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Abstract: Epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKI) have been employed as the first-line treatment for lung adenocarcinoma patients with advanced EGFR-positive mutations, especially in patients with *EGFR* exon 21 L858R mutation or an exon 19 deletion. However, almost all patients inevitably develop acquired resistance to EGFR-TKI after 1 to 2 years, due to *EGFR* gene secondary mutations, *MET* gene amplification, *KRAS* mutation, loss of phosphatase and tensin homolog (PTEN), and other mechanisms. Therefore, the EGFR-TKI acquired resistance becomes a bottleneck of continuation for EGFR-TKI therapy in clinic. The neuronally expressed developmentally downregulated 4 (NEDD4), an E3 ubiquitin ligase, has been demonstrated to play an important role in the development and progression of human cancers. NEDD4 is frequently overexpressed in non-small cell lung cancer carcinomas (NSCLC) and is implicated in the regulation of ubiquitination of Ras and PTEN. NEDD4 overexpression promoted cellular transformation and induced EGFR-TKI acquired resistance in NSCLC. In this review, we will describe how NEDD4 regulates EGFR-TKI acquired resistance in NSCLC, and further discuss its mechanism, including PTEN poly-ubiquitination, Ras signaling activation, and EMT conversion. Therefore, targeting NEDD4 could be a potential therapeutic strategy for NSCLC after EGFR-TKI acquired resistance.

Keywords: NEDD4, non-small cell lung cancer, ubiquitination, EGFR-TKI, resistance

Introduction

Lung cancer is the most common malignant tumor with the highest morbidity and mortality worldwide, and is clinical broadly classified into two pathological categories: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC is further subdivided into squamous cell carcinoma and adenocarcinoma, which accounts for approximately 85% of all lung cancer. In addition, the prognosis of lung cancer is still not very satisfactory after surgery, standard chemotherapy or radiotherapy [1]. The 5-year overall survival rate of patients with advanced NSCLC is still low, only ranging from 10% to 15% [2]. Such disappointing results drive us to pursue new therapeutic strategies.

Epidermal growth factor receptor (EGFR), a transmembrane receptor expressed by oncogene c-ErbB, is one of the molecular target for NSCLC therapy. The extracellular domain of EGFR binds to EGF to activate the intracellular domain of tyrosine kinase and it triggers the phosphorylation of proteins in the PI3K/AKT/mTOR, Ras/Raf/MAPK, JAK/STAT signaling pathways, which mediates cell survival and proliferation, migration and invasion, and is involved in angiogenesis when mutated [3]. Many published investigations report that EGFR is overexpressed in 45 to 85% of NSCLC patients, which is closely related to the percentage of poor prognosis and resistance to radiotherapy and chemotherapy. Therefore, it is an important molecular target for lung cancer therapy.

Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI), mainly quinoline compounds, inhibit the intracellular tyrosine kinase domain of EGFR by competitively binding with ATP, which leads to cell cycle arrest in G1 phase and an increase in apoptosis of tumor cells by blocking the pathway of cellular signal transduction [4]. NSCLC patients with mutations of the *EGFR* gene involving exon 19 dele-

tion or the L858R point mutation in exon 21 are highly sensitive to EGFR-TKI. Clinical data show that considerable improvement has been made by EGFR-TKI treatment with over a 70% response and almost one-year progression-free survival in patients with active mutations of *EGFR* gene. For this reason, EGFR-TKI was approved for first-line treatment of NSCLC patients with activating mutations in the *EGFR* gene. However, most patients will inevitably develop acquired resistance to EGFR-TKI with 12 months [5]. Resistance to EGFR-TKI includes various mechanisms, such as the secondary mutation (T790M), amplification of c-met, *RAS* mutation, loss of PTEN, and other mechanisms.

NEDD4, a HECT E3 ubiquitin ligase, is a product of the Neural Precursor Cell-Expressed Developmentally Downregulated gene 4 and exerts its biological function mainly by regulation of protein degradation in an ubiquitination-dependent manner. Protein ubiquitination is a post-transcriptional modification process, which mediates a range of cell processes including neuronal development, tumorigenesis and progression. Recent studies report that NEDD4 is overexpressed in multiple types of solid tumors including NSCLC [6, 7], colorectal cancer [8], gastric carcinomas [9], breast cancer [10], and bladder cancer [11]. Amodio et al. reported that NEDD4 was overexpressed in 80% of lung tumors in 103 NSCLC resections [6]. In fact, overexpression of NEDD4 facilitates the tumorigenicity and progression of lung cancer. Here we review the role and regulation of NEDD4 in the EGFR-TKI acquired resistance of NSCLC and discuss the identification of new biomarkers and treatment strategies for EGFR-TKI resistance in NSCLC.

NEDD4 ubiquitination regulates PTEN in EGFR-TKI acquired resistance

The PTEN gene, located on chromosome 10q 23.3, encodes a 53 kDa dual-specificity lipid and protein phosphatase [12]. The activity of lipid phosphatases is intimately related to the role of PTEN as a tumor suppressor by inhibiting phosphatidylinositol 3-kinase (PI3K)/AKT signal pathway activation. The phosphatidylinositol 3,4,5-trisphosphate (PIP3) is produced though the phosphorylation of phosphatidylinositol 3,4-bisphosphate (PIP2) by activation of PI3K, which represents the activated status. PTEN hydrolyzes the phosphatase group at D3 position of the PIP3, resulting in PIP2 formation and consequently inhibiting PI3K activation [13]. In particular, the deficiency of PTEN function in tumor cells is associated with malignance, poor clinical outcome and resistance to anticancer drugs [14-17].

The activity of PTEN is regulated by post-translational modification including phosphorylation and ubiquitination [18, 19]. NEDD4 [20], WWP2 [21], and X-linked inhibitor of apoptosis protein (XIAP) [22] are all reported to be ubiquityltransferase E3 ligases of PTEN. Prior study has indicated that PTEN is found in cell nuclei, and the nuclear PTEN is essential for tumor suppression. The mechanism of PTEN nuclear import is mediated by its mono-ubiquitination at K13 and K289 sites [23]. In contrast, PTEN poly-ubiquitination mediated by NEDD4 leads to PTEN protein degradation in cytoplasm. Thus, PTEN ploy-ubiquitination is associated with tumorigenesis [20]. Interestingly, nuclear transport of PTEN may protect PTEN form poly-ubiquitination through NEDD4. Therefore, NEDD4 mediates protection and degradation of PTEN by its mono-ubiquitination and poly-ubiquitination, respectively.

The downregulation of PTEN protein levels is up to 70% in NSCLC tumors. This downregulation can lead to PTEN inactivation and resistance to EGFR-TKI *in vitro* and *in vivo* studies as well as in NSCLC patients [24, 25]. In addition, the survival rates of NSCLC patients with activating mutation of *EGFR* gene and low PTEN expression were lower than those with higher PTEN expression [26]. Downregulation of PTEN through ubiquitin-mediation is considered to be the main mechanism of loss of PTEN function. Furthermore, NEDD4 is a proto-oncogenic ubiquitin ligase for PTEN, which is overexpressed in 80% of NSCLC patients and negatively regulates PTEN abundance by ubiquitin-mediated degradation of PTEN [6]. Recently, some evidence indicates that NEDD4 regulates PTEN protein levels in acquired resistance to EGFR-TKI by activation of AKT and EGFR [7, 27]. Inhibition of the PI3K/AKT signal pathway can be an effective treatment strategy to NSCLC with acquired resistance to EGFR-TKI due to PTEN loss [12, 28]. It has also been observed that NEDD4 overexpression correlated with decreased levels of PTEN protein and increased

AKT activation in lung cancer, whereas knock-down of NEDD4 expression inhibited PTEN ubiquitination and increased PTEN protein levels [6]. Sun et al. have demonstrated that NEDD4-mediated PTEN downregulation plays an important role in the resistance to erlotinib in HCC827/ER cells. Knock-down of NEDD4 expression inhibited PI3K/PTEN/AKT signal activation and reversed the resistance to erlotinib partially [7]. Taken together, NEDD4 promotes the acquired resistance to EGFR-TKI though poly-ubiquitination for decreasing PTEN protein expression in NSCLC.

NEDD4 mediates Ras ubiquitination in EGFR-TKI acquired resistance

Ras proteins, including K-Ras, H-Ras, N-Ras, are small guanosine triphosphatases (GTPases) that are molecular switches for regulating a wide range of cellular activities, especially in controlling cell proliferation, differentiation, migration and invasion [29]. Several investigations have reported that Ras plays a central role in driving oncogenesis in human tumors [30]. In NSCLC, mutations in the *Ras* gene occur with a frequency of about 30%, and the single point mutation of Ras is sufficient to trigger tumor malignant transformation [31]. Ras mutations induce a persistent activation state of Ras due to defect in hydrolysis ability of GTP to GDP, leading to a constitutive activation of subsequently kinases associated with RAF/MAPK proteins and transcription factors such as MYC, FOS and JUN [32]. In addition to the oncogenic activation by *Ras* gene point mutations, Ras signaling can determine the inefficiency of gefitinib in therapeutic doses [33], so the mechanism of resistance is associated with tumor cells independent of the signals from the EGFR.

A recent study indicated that NEDD4 targeted Ras protein for ubiquitination and degradation [34]. Wide-type NEDD4 but not NEDD4 C867A mutant significantly decreased both the endogenous and exogenous Ras protein by ubiquitination, suggesting NEDD4 as an E3 ubiquitin ligase for Ras. Ras small GTPases are well-known proto-oncoproteins, which might be involved in controlling tumorigenesis. Knock-down of NEDD4 inhibited cell colony-formation in soft agar; accordingly, overexpression of NEDD4 markedly increased cell colony-formation [34]. Recent investigation showed that Ras stability and activity were regulated by ubiquitination. In particular, ubiquitination of Gly-12-Val mutation of K-Ras increased its ability to bind the downstream effectors PI3K and Raf [35]. It could be beneficial that pharmaceutical disruption of mono-ubiquitination of K-Ras in treating cancers with Ras mutations. Kim et al. reported that H-Ras interacted with the WD40 domain of β-TrCP, which promoted H-Ras ploy-ubiquitination and degradation. Ras-mediated cellular transformation was also inhibited by the overexpression of β-TrCP [36]. However, Ras mutants constantly activate downstream of EGF signaling pathways, acting as an oncogenic factor for cancer development. NEDD4 mediated Ras degradation in normal cells in response to upstream growth signals. In addition, Ras activation up-regulated NEDD4 expression, and NEDD4 was essential in inhibiting transformation and tumorigenesis through Ras ubiquitination. However, activating mutations of Ras, especially on Gly-12-Val, blocked NEDD4-mediated Ras degradation, which might be essential for Ras-driven tumorigenesis [34]. Moreover, Ras mutations led to NEDD4 overexpression by sustained Ras activation, thereby establishing a feedback loop mechanism between NEDD4 and Ras signaling. Importantly, the upregulated NEDD4 levels enhanced NEDD4-mediated PTEN degradation in cancer cells, therefore leading to EGFR-TKI resistance in NSCLC. Hence, interruption of this regulatory loop mechanism may impair Ras signaling and EGFR-TKI resistance.

NEDD4 mediates EMT in EGFR-TKI acquired resistance

Epithelial to mesenchymal transition (EMT) is a physiological process in embryogenesis and wound healing, but also occurs in tumor progression [37]. EMT is characterized by the combined loss of epithelial phenotypic proteins, such as E-cadherin, claudin and occluding, and the strong elevation of mesenchymal markers, such as N-cadherin and vimentin [37]. In the process of EMT, epithelial cells lose their polarity and adhesion, gain a mesenchymal phenotype, and become more migratory and invasive. Furthermore, EMT leads to up-regulation of anti-apoptotic signals inducing tumor cells to be malignant and less responsive to treatment [38].

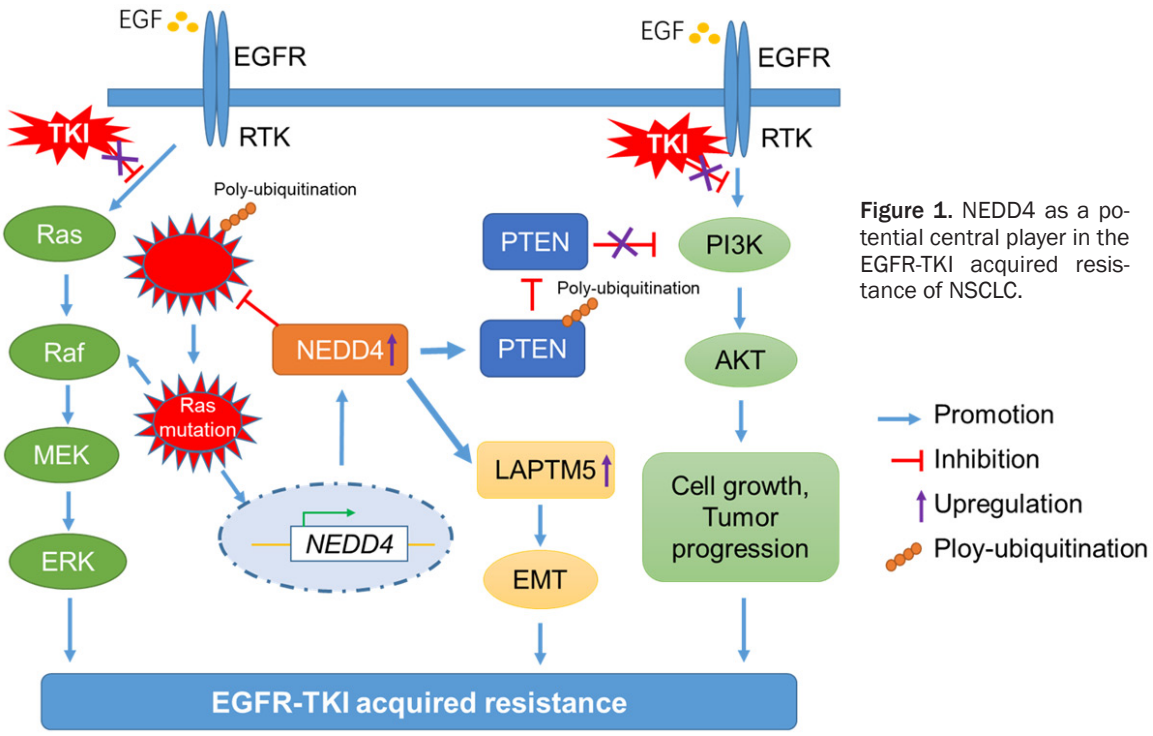

Figure 1. NEDD4 as a potential central player in the EGFR-TKI acquired resistance of NSCLC.

The EMT status has been investigated as a marker in both prognosis of NSCLC and as a predictor of therapeutic response. Particularly, EMT plays an important role in the process of EGFR-TKI acquired resistance in NSCLC. The detection rate of EMT in NSCLC patients with EGFR-TKI resistance was 20% in clinic [39]. Weng et al. reported that NSCLC cells with acquired resistance to gefitinib or osimertinib exhibited EMT phenotype, with a decrease in E-cadherin, and increase in vimentin; leading to resistant cells with enhanced ability of migration and invasion. Knock-down of E-cadherin in parental cells increased resistance to gefitinib. However, overexpression of E-cadherin led to restoration of gefitinib sensitivity [40]. Vazquez-Martin et al. reported that PC-9 cells with acquired resistance to erlotinib were associated with EMT phenomenon. The mesenchymal status-related activation of IGF-1R was ultimately responsible for the acquired resistance to erlotinib in *delE746-A750*-mutated PC-9 cells [41]. Indeed, the resistant cells exhibit not only EMT features but also stem cell-like properties, and are associated with EGFR-TKI resistance [42].

Recent studies have found that NEDD4 is involved in EMT that is closely associated with chemoresistance in human malignancies. Feng et al. reported that upregulation of NEDD4 was observed to relate to EMT in radiotherapy and adjuvant cisplatin chemotherapy resistant nasopharyngeal carcinoma cells. Moreover, depletion of NEDD4 in resistant cells led to a partial reversion of EMT phenotype and chemoresistance to cisplatin [43]. In lung cancer, miRNA-93 is involved in TGF-induced EMT. Particularly, miRNA-93 bound directly to the 3'-UTR of the NEDD4-like E3 ubiquitin ligase messenger RNA, leading to downregulation of NEDD4L expression at the protein level and promoting EMT though TGF-signaling [44]. NEDD4 has been reported to upregulate expression of lysosomal-associated multi-spanning membrane protein 5 (LAPTM5), which promoted EMT though phosphorylated ERK1/2 and p38MAPK. In addition, down-regulation of NEDD4 inhibited tumor cell proliferation and viability via decreasing LAPTM5 expression [45]. Since several studies have reported that EMT was involved in cancer cell migration and invasion, we conclude NEDD4 mediated cell migration and invasion in human cancers. Zhang et al. reported that NEDD4 promoted glioma cell migration and invasion though triggering ubiquitination of cyclic nucleotide Ras guanine nucleotide exchange factor (CNrasGEF) [46].

Shao et al. indicated that NEDD4 interacted with EGFR and participated in EGFR-signaling-dependent lung cancer migration. Furthermore, NEDD4 mediated the lung cancer cell migration and invasion though activation of the lysosomal cathepsin B secretion pathway [47]. Song et al. also demonstrated that knock-down of NEDD4 decreased proliferation, migration, and invasion and improved chemosensitivity to cisplatin and paclitaxel in lung adenocarcinoma by regulation of PI3K/AKT pathway [48]. Collectively, NEDD4 is an essential E3 ubiquitin ligase in maintaining the EGFR-TKI resistant phenotype by EMT signaling.

Conclusions and future perspectives

NEDD4 is a member of the E3 ubiquitin ligases and functions in the ubiquitin proteasome system, which is a critical regulatory factor of diverse cellular pathways for tumor initiation, progression, migration and resistance to anticancer therapies. In lung cancer, it has been observed that NEDD4 is overexpression in 80% of NSCLC tumor tissues and correlated with the loss of PTEN protein. In addition, overexpression of NEDD4 promotes EGFR-TKI acquired resistance in NSCLC. The mechanism of EGFR-TKI resistance is involved with NEDD4 leading to PTEN ploy-ubiquitination, Ras signaling activation and EMT conversion (**Figure 1**). Collectively, the role of NEDD4 in EGFR-TKI acquired resistance suggests that NEDD4 could be a potential therapeutic target for treatment of human NSCLC.

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Disclosure of conflict of interest

None.

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