Foxm1 Transcription Factor Regulates Lung Adenocarcinoma Development

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Abstract. Foxm1 is a potential transcription factor that is abundantly expressed in highly proliferative human lung cancer cells. Foxm1 also strongly correlates to lung fibrosis, epithelial-mesenchymal transition (EMT) to enhance metastatic program during the lung adenocarcinoma development. Foxm1 plays a pivotal role in controlling cell cycle phase through the G1-S-G2 checkpoint. The increasing Foxm1 and K-ras oncogene expression significantly associate with tumor growth and poor prognosis that potentially modulate patient’s mortality in a subject with lung carcinoma. The genetic evidence showed that the silencing of Foxm1 resulted in the decrease in lung tumorgenesis. Thus, Foxm1 may contribute in the future as the potential target for cancer therapy by reducing lung fibrosis, EMT, and tumor cell proliferation to improve patient’s survival rate.

Keywords: Foxm1, lung adenocarcinoma, transcription factor, prognosis

1. Introduction
Lung adenocarcinoma (LUAD), the leading subtype of non-small cell lung cancer (NSCLC), causes over 500,000 deaths per year worldwide [1, 2]. In Caucasian lung cancer patients, oncogenic Kras is one of the major causes of NSCLC, and there is no effective chemotherapy agent available for cancers harboring oncogenic Kras mutations [3]. Hence, identifying new downstream molecular targets of K-RAS signaling is critical for improving current therapeutic outcomes of lung cancer patients. Many reports indicated that KRAS/MAPK signaling activated downstream Forkhead box m1 (Foxm1) transcription factor that regulated transcription network of genes essential for cell cycle as well as cell migration, angiogenesis, epithelial to mesenchymal transition (EMT), stemness during embryonic development, injury repair, and cancer progression [4-12].

2. Conditional deletion of Foxm1 inhibited carcinogens induced lung tumorigenesis
We previously showed that FOXM1 levels increased in the majority of solid human cancers, including NSCLC [10]. Clinical evidence showed that the high expression level of FOXM1 associated with poor prognosis in NSCLC patients [13]. Consistent with our research results from Rosa26-FOXM1 transgenic mouse model, ubiquitous expression FOXM1 in all mouse tissue promoted the growth of
lungs caused by tobacco carcinogens 3-methylcholanthrene (MCA)/butylated hydroxytoluene (BHT), a well-known lung tumor initiation/promotion protocol [14]. In contrast, while using doxycycline (Dox)-inducible CRE/loxP system to conditionally delete Foxm1 floxed gene from lung type II epithelial cells of SPC-rtTA/tetO-Cre/Foxm1fl/fl mice (epFoxm1−/−) [15], we demonstrated that conditional deletion of Foxm1 alleles from lung epithelial cells caused resistance to tumorigenesis induced by tobacco carcinogens urethane or MCA/BHT [16]. To further determine whether Foxm1 is involved in lung tumor progression and maintenance, we thus conditionally deleted Foxm1 alleles in pre-existing lung adenomas (induction with urethane protocol) or adenocarcinoma (induction with MCA/BHT protocol), and found that inhibition of Foxm1 expression delayed the growth and expansion of these lung tumors [16], suggesting that Foxm1 is critical for tumor progression and can be a promising target for anti-tumor therapy.

We also demonstrated that the decreasing lung tumorigenesis in epFoxm1−/− mice was associated with a reduced level of Topoisomerase-2α (TOPO-2α), which is consistent with the result of FOXM1 siRNA-transfected A549 cells. TOPO-2α is a ubiquitous enzyme that plays an essential role in regulating DNA under- and over-winding as well as resolving knots and tangles in the genetic material through transient double-stranded breaks [17]. We further showed that Foxm1 protein binds TOPO-2α gene promoter and activated gene expression by chromatin immunoprecipitation (ChIP) assay and promoted dual luciferase assay respectively. Altogether, these results indicated that deletion of Foxm1 from respiratory epithelial cells is sufficient to reduce proliferation of tumor cells and chemically decrease induced lung tumorigenesis.

3. Conditional deletion of Foxm1 prevented oncogenic K-ras driven lung tumor initiation

Published studies reported that the expression of the activating KrasG12D in distal lung epithelium disrupted branching lung morphogenesis by causing abnormal localization of phospho-ERK in distal lung tubules[18, 19]. Conditional deletion of Foxm1 from distal epithelial cells in embryonic lungs of SPC-rtTA/TetO-Cre/TetO-KrasG12D/Foxm1fl/fl mouse, attenuating the lung branching defects caused activating Kras; suggesting Foxm1 is a major effector of Ras/MAPK signaling during distal lung epithelium branch morphogenesis in vivo [20]. As urethane-induced lung tumors frequently carry gain-of-function mutation on Kras that is a frequent driver oncogene in human NSCLC [21, 22], we, therefore, investigated whether deletion of Foxm1 would prevent lung tumor formation caused by the activated K-Ras by using K-RasG12D lung cancer model. We used SPC-rtTA/TetO-CreG2/TetO-KrasG12D/Foxm1fl/fl mice (epKras/epFoxm1−/−) and SPC-rtTA/TetO-KrasG12D/Foxm1fl/fl (epKras) control littermate for lung cancer studies. In the presence of Dox, the reverse tetracycline transactivator (rtTA) bound to the TetO promoter, resulted in expression of activating Kras to induce lung tumor formation in epKras mice; while in the epKras/epFoxm1−/− mice, the induced Cre recombinase caused deletion of the floxed Foxm1 exons 4–7 that encode DNA binding and transcriptional activation domains, along with K-RasG12D expression. The computer-based three-dimensional reconstruction of Micro-Computed Tomography (micro-CT) scan imaging of whole mouse lungs was performed, and we demonstrated that deletion of Foxm1 from KrasG12D expressing respiratory epithelium (epKras/epFoxm1−/−) dramatically reduced the number and size of lung tumors. Moreover, chromatin immunoprecipitation (ChIP) assays identified FOXM1 directly binds and transactivates Ikbkb, Nfkb2, N-Myc, Pttg1, and Cdkn2a gene promoters, suggesting the loss of Foxm1 inhibited expression of K-Ras target genes that are critical for the nuclear factor-kB (NF-kB) and c-Jun N-terminal kinase (JNK) pathways. This result showed that Foxm1 is required for tumor initiation and proliferation of Kras gain-of-function mutant lung epithelial cells [23].

To further investigate whether Foxm1 could be a potential therapeutic target for lung cancer harboring oncogenic Kras, we next tested the role of Foxm1 in lung tumor maintenance and progression. After five months of Dox treatment to CCSP-rtTA/TetO-KrasG12D/Foxm1fl/fl mice, the lung tumors were formed and confirmed by micro-CT imaging. These lung tumor-bearing mice were then intratracheal injected (IT) with Cre-expressing adenovirus (Ad-Cre) to induce Foxm1 gene alleles deletion in mouse lung tumors followed by an additional micro-CT examination. We found that IT
injection with Ad-Cre to CCSP-rtTA/tetO-Kras^{G12D}/Foxm1^{fl/fl} mice that bear KRAS^{G12D}-induced lung tumors, causing 75% of preexisting lung tumor regression. Genetic deletion of Foxm1 alleles in lungs resulted in Kras^{G12D} lung tumor regression associated with a reduction of cell proliferation and an increase in apoptosis. Moreover, *in vitro* disruption of FOXM1 expression by shRNA resulted in diminished cell proliferation on a culture plate and anchorage-independent growth in soft agar of KRAS-mutated lung cancer NCI-H23 as well as the KRAS^{G12D}-transformed BEAS-2B cells. Accordingly, these results demonstrated that Foxm1 is critical for oncogenic KRAS signaling pathway in both maintenance and progression of LUAD.

4. Targeting Foxm1 suppressed oncogenic EGFR-driven lung tumor development

Epidermal growth factor receptor (EGFR)-mediated signaling is critical for cell proliferation, migration, and survival. In East Asia, 30-55% percent of human NSCLC is highly associated with the gain of function mutations in the EGFR gene [24]. In Taiwan, specifically, 90% of female lung cancer patients are non-smokers, and 50% of them harbor EGFR gene mutations or polymorphisms [25]. In the absence of extracellular stimuli, oncogenic EGFR tyrosine kinase activates downstream signaling pathways, including JAK-STAT3, Ras-Raf-MAPK, PI3K-AKT, leading to cell proliferation and transformation [26]. In the past decade, the development of the first- to third- generation EGFR tyrosine kinase inhibitors (TKIs) consequently proved that targeting EGFR was an effective therapy in EGFR-mutant LUAD [27,28]. However, current EGFR-TKI chemotherapy often causes drug-resistant tumor recurrence within six months, which limits the range of possibilities for subsequent treatments and impacts on patient outcomes.

We have retrospectively recruited 123 patients diagnosed with LUAD at National Taiwan University Hospital Hsinchu branch, and the expression levels of FOXM1 and EGFR in these lung cancer biopsies were detected by immunohistochemical (IHC) staining followed by correlation analysis with patients’ clinical characteristics. We found that FOXM1 and EGFR expression levels were significantly positively correlated. LUAD tissues with high FOXM1 expression levels were significantly correlated with advanced clinical stages (stage III/IV) of patients, which was also associated with shorter overall survival (OS) of patients. In both groups of patients with EGFR mutations or specific EGFR p.L858R mutation, patients with high FOXM1 expression showed shorter OS as compared to low FOXM1 levels patients. Recent *in vitro* studies indicated Foxm1 was a transcription target of STAT3, a downstream target of EGFR signaling. Similarly, we found that inhibition of EGFR activity by TKIs, Erlotinib or Osimertinib, resulted in suppressed FOXM1 levels in LUAD cell lines harboring EGFR-mutation; suggesting FOXM1 was a downstream target gene of EGFR signaling pathway *in vitro*.

The established mouse lung cancer models have shown that conditional expression of activating mutant EGFR was sufficient to induce lung adenocarcinoma formation, which demonstrated the importance of EGFR in human lung carcinogenesis [29, 30]. We, hence, determined whether Foxm1 gene was essential for EGFR induced lung tumorigenesis by using CCSP-rtTA^{gr}/TetO-Cre^{gr}/TetO-EGFR*L858R^{wt}/Foxm1^{fl/fl} mice (C/EGFR/Foxm1^{-/-}) and control CCSP-rtTA^{gr}/TetO-EGFR*L858R^{wt}/Foxm1^{fl/fl} (C/EGFR/Foxm1^{fl/fl}) littermates for the experiment. After Dox treatment for 3.5 months, airway epithelial cell-specific expression of rtTA protein, thus, activated expression of EGFR^{L858R} and mediated Foxm1 gene deletion. EGFR^{L858R} lung tumors were visualized and associated with enlarged lung lobes of C/EGFR/Foxm1^{-/-} mice when they were compared to C/EGFR/Foxm1^{fl/fl} mice and Foxm1^{fl/fl} control. Micro-CT imaging and pathological staining revealed severe lung tumorigenesis in C/EGFR/Foxm1^{fl/fl} lungs compared to tumors found in C/EGFR/Foxm1^{-/-} lungs; while none tumor was detected in the control Foxm1^{fl/fl} mice. The significantly decreasing nodule loadings in C/EGFR/Foxm1^{-/-} were correlated with a significantly increasing lifespan when they were compared to their tumor-bearing C/EGFR/Foxm1^{fl/fl} littermates. The reduction of cell proliferation in C/EGFR/Foxm1^{-/-} lung was determined by IHC for cell proliferation markers Ki-67, which was consistent with the reduced proliferation in Foxm1-depleted human LUAD cancer cell lines harboring EGFR mutations.
Accordingly, these results demonstrated that conditional deletion of Foxm1 gene from airway epithelial cells inhibited lung tumorigenesis induced by chemical carcinogens, oncogenic EGFR or Kras, indicating Foxm1 is is a critical transcription factor for lung cancer development.

5. Conclusion
The future development of cancer therapy based on the molecular target may potentially be addressed on the primary contributor that involved in the essential signalling pathway during lung tumorigenesis. Importantly, Foxm1 shows a vital contribution to lung cancer development and induce the acceleration of tumor growth, fibrogenesis, and metastasis. Hence, Foxm1 suggests the novel target for lung cancer therapy and may contribute to improving clinical diagnosis in a patient with lung adenocarcinoma.

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