A New Mechanism for Foxm1 to Enhance the Malignant Phenotype of Lung Cancer Cells: Increasing Egfr Transcription and Activating Egfr Signaling Pathway

Chia-Jui Chen

Liberty Christian Academy, Virginia, United States

852343722@qq.com

Keywords: Foxm1, Egfr, Lung cancer, Transcription

Abstract: Identification as an important regulator of a variety of cancers, especially lung cancer, Forkhead Box M1 (FOXM1) is widely expressed in the recurrence of lung cancer drug resistance. However, the character of FOXM1 in the recurrence of lung cancer resistance still needs further exploration. Here, we explored the genetic expression profile of cells after overexpression of FOXM1. By analyzing differentially expressed mRNA, we found that target genes related to EGFR and its signaling pathway downstream in lung cancer cells are up-regulated, and that the up-regulation is relative to the malignant phenotype of tumor cells induced by FOXM1 overexpression, which is characterized by accelerated cell division and proliferation and promotive sphere formation ability. FOXM1 increases the expression of EGFR and promotes cell proliferation. In terms of mechanism, FOXM1 promotes the expression of EGFR, leading to a promotion in its phosphorylation level, which greatly activates its downstream signal transduction, and finally promotes cell division and growth.

1. Introduction

It is fairly well known that lung cancer has become the most lethiferous cancer disease and causes the highest mortality of all cancer in the whole wide world. Each year, there are about 1.8 million people who are diagnosed with lung cancer; even more shocking, 1.6 million of them died from this deadly cancer [1]. Many epidemiologists are highly focused on studying mechanisms of oncogenicity effect and working hardly to research and try to remove the factors that induced tumor recurrence. Moreover, those epidemiologists have devoted an enormous amount of energy to deal with medical resistance in clinical patients. In order to effectively eliminate the potential threats to treat lung cancer patients, some experts attempt to explore the mechanism of tumorigenicity. In many clinical practices, researchers have focused their research on the signaling pathway proteins that could operate the proliferation of cancer tumors. Among many signaling pathways, EGF/EGFR signaling pathway is the key point that many experts lay emphasis on. In tumor cells, the epidermal growth factor receptor (EGFR/ErbB/HER) family members are always abnormally expressed, especially EGFR [2]. As a result, EGFR becomes a brilliant focus and target in cancer development, cancer treatment and metastatic progression.

Before discussing the fundamental functions of EGFR, it is necessary to emphasize the crucial role of EGF in the signaling pathway functions performed by the binding of EGFR. Epidermal growth factor (EGF) is a type of multifunctional glycoprotein, playing an essential character in regulating cell growth, proliferation, and differentiation. EGF can be observed in several body fluids of animals, such as milk, urine, intestinal fluid, and other fluids that can be produced by certain body organs. In our body, kidney is the primary organ that produce EGF; the prostate fluids contain the the highest concentration of EGF, but urine was discovered that possess high expression level of EGF. By binding to EGFR, they are able to stimulate receptor tyrosine kinase (RTK), which can regulate cellular signaling pathways. EGF is diffusely expressed in the body organisms and regulates tissue development, regeneration, and ion transport of the organism. [3] These effects could be operated either by EGF itself or by binding EGFR and with other members of the ErbB

  DOI: 10.25236/bmmss.2021.003

family. The gland EGF activates EGFR to activate the downstream signaling pathways, which are protein molecules RAS-RAF-MEK-MAPK and PI3K-AKT.

FOXM1 is a mammalian transcriptional factor that modulates the process of normal cell proliferation [5]. However, it is circularly overexpressed in various human cancer. In oncogenesis, the overexpression of FOXM1 in cancer cells has already been observed in varieties of cancer, indicating that FOXM1 is necessary in tumorigenesis. Generally, FOXM1 induces tumor development by the stimulating transcriptional of its targets [6]. In tumor cells, FOXM1 is normally overexpressed and increased stably when interacting with several certain proteins or RNAs. Moreover, FOXM1 could also be the facilitator to activate and enhance other property of carcinogenic pathways. As a result, many researchers also pay close attention to deal with the function of FOXM1 and design new FOXM1 inhibitors that are able to suppress the interaction of FOXM1 and other proteins or RNAs [7].

When EGFR is activated by EGF, it activates signaling molecules to conduct signals from outside to inside the cell membrane. [3] Based on the high level of EGFR expression in tumor cells, a series of anti-tumor medication have been developed and put into practice. According to the results of clinical experiments, tumors and cancer patients will develop certain medical resistance after using drugs that can inhibit the expression of EGFR, and we need to understand the reasons and try to solve the problem of medical resistance in tumors and cancers. Our preliminary results suggested that EGFR may be regulated by transcriptional mechanisms so that it is overexpressed in lung cancer, but we still have no idea with the exact mechanisms. Therefore, our framework of this study is to solve the transcriptional mechanism of EGFR.

2. Materials and Experimental Methods

2.1 Cell Lines.

We purchased PC9 cells from the American Type Culture Collection (ATCC). Then we cultured the PC9 Cells in RPMI-1640 medium with 10% FBS, 5% CO2 at 37 °C.

2.2 Cck-8 Assay Analysis.

Count the number of cells in the prepared cell suspension with a cytometer first, and then inoculate the cells into the culture plate. According to the ratio (for example: 1/2 ratio), use the medium to dilute to a cell concentration gradient in sequence. Generally, 3-5 cell concentration gradients are required, and 3-6 replicate holes are recommended for each concentration. After inoculation, incubate for 2-4 hours to make the cells adhere to the wall, then add CCK reagent to incubate for a certain period of time and then measure the OD value, and make a standard curve with the number of cells as the abscissa (X axis) and the OD value as the ordinate (Y axis). . According to this standard curve, the number of cells in unknown samples can be determined.

2.3 Western Blot Analysis.

The well-growing cells were harvested and the lysate was cleaved. The protein liquid was collected and measured the protein concentration. Constant voltage electrophoresis, membrane-constant current 276 mA, wet rotation 120 minutes. After that, it was closed for 2 hours, added one antibody, shaking overnight, incubated at room temperature for 1 hour with HRP labeled the second antibody, and exposed the PVDF film to the exposure machine after dropping ECL luminescent solution.

2.4 Rt-Pcr and Qrt-Pcr.

Cells were first homogenized in Trizol (Takara) and total RNA was isolated according to the manufacturer’s instructions. Total RNA (1000 ng) was detected by a SYBR Green One-Step RT-qPCR Kit (Takara, RR037A; Vazyme) as described in the manual. The PCR primers for the 18s, FOXM1 and EGFR mRNA, the real-time PCR was performed using the Applied Biosystem 7500 Fast PCR System. The mRNA levels were normalized against 18s mRNA.

2.5 Rna-Seqencing and Gsea Analysis

RNAs isolated from construted FOXM1 overexpressing and vector control cells (built in our lab previously) were sequenced by HiSeq2000 (Illumina). RNA-seq were evaluated using FastQC (version 0.10.1). We used GSEA (v2.0.13) to assess enrichment of gene signatures related to EGFR TARGET GENES in EGFR.

3. Migration Assays

Transwell (Costar, Corning, NY) was performed to determine the cells migration. In brief, 4 × 104 cells were seeded onto the top chamber and DMEM containing 10% FBS was added to the bottom chamber as a chemoattractant. After a 24-h incubation, the cells migrated through the membrane (migration) were stained with crystal violet and counted in 5 fields. Images were taken using ImageJ software (Bethesda, MD, USA). Each experimental group was repeated three times.

3.1 Immunofluorescence

Cell climbing tablets were placed in a 24-well plate. 30,000 cells from each group were plated in 24-well plate. In complete medium, the cells were cultured for one night. removed the culture medium, and cleand the PBS. Adding methanol for 15 minutes, after fixing, Tritone penetrated the membrane for 10 minutes, goat serum was blocked and then added the corresponding the first antibody. After washing first antibody FOXM1 and EGFR, the fluorescent second antibody was added. After washing the second antibody, visualized using a confocal microscope (Leica, Wetzlar, Germany).

3.2 Statistics.

Data were analyzed with GraphPad Prism. The non-paired Student t test was performed to analyses the comparison of two groups. When $P < 0.05$, the difference was significant statistically.

4. Result

4.1 The Association between Foxm1 and Egfr Signaling Pathway

In order to study the downstream molecules regulated by FOXM1, we established FOXM1 overexpressing cell line for performing RNA-Seq assay. The control group was an empty vector. We found that after FOXM1 was overexpressed, the mRNA level of EGFR was significantly increased (Fig 1A), indicating that FOXM1 is EGFR Potential transcriptional regulator. In order to further clarify whether FOXM1 is involved in the regulation of EGFR downstream pathways, we conducted a Gene set enrichment analysis (GSEA), GSEA revealed obviously enrichment patterns of gene signatures related to EGFR TARGET GENES in EGFR (Fig 1B). These results have verified that FOXM1 could be possible involved in regulating EGFR pathway.

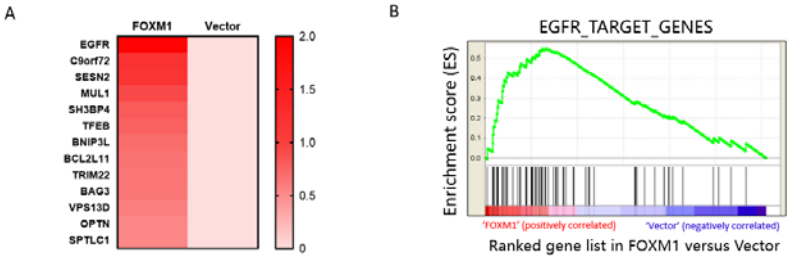

Fig.1 Foxm1 Could Be Possibly Involved in Involved in Regulating Egfr Pathway.

A.Gene alteration expression profile in FOXM1 overexpressing cells.
B.Using GSEA to analyze the enrichment of differential genes in FOXM1 overexpressing cells.

4.2 Foxm1 Positively Regulates Egfr Gene Expression

To further investigate and verify whether FOXM1 would regulate EGFR transcription, several FOXM1 overexpressed cell lines were successfully established. We found that overexpression of FOXM1 significantly upregulated EGFR (Fig 2A). Conversely, downregulation of FOXM1, which represent the inhibition of FOXM1, expressively down-regulated or inhibit EGFR expression (Fig 2B). Western Blot results have displayed that the down-regulation of FOXM1 significantly reduced EGFR protein levels (Fig 2C). In order to confirm our results, the immunofluorescence assay (IF) was implemented to analyze the expression of FOXM1 and EGFR. The actual outcome showed that the expression of EGFR also increased in cells with high FOXM1 expression (Fig 2D). The final results suggested that FOXM1 is able to regulate the expression of EGFR at the transcriptional level, thus affecting the protein level of EGFR.

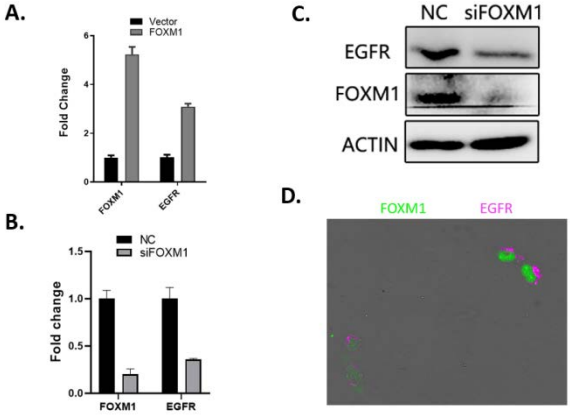

Fig.2 Foxm1 Regulates the Expression of Egdr in Transcriptional

A. Overexpression of FOXM1 enhances EGFR mRNA levels

B. Knockdown of FOXM1 expression reduces EGFR mRNA levels

C. Knockdown of FOXM1 expression reduces EGFR proteinA levels

D. Fluorescence staining results of FOXM1 and EGFR. The FOXM1 marked by green is mainly located in the nucleus. Purple labeled EGFR, mainly located in the cell membrane.

4.3 Up-Regulation of Egfr Was Involved in Foxm1-Induced Tumorigenicity

To determine whether FOXM1 promotes tumor development through EGFR, we have perfectly constructed FOXM1-overexpressing cell lines, and short interfering RNA is used to knock down EGFR. In the Western Blot experiment, results showed that EGFR was effectively inhibited by us (Fig 3A). We next created the cell growth curve at the bottom, which indicates that FOXM1 can significantly enhance the cell growth rate, but this effect can also be inhibited by the interference or knockdown of EGFR (Fig 3B). By adopting Transwell Assay, it has shown that overexpression of FOXM1 enlarged the metastasis of tumor cells, while after EGFR was silenced, the tumor metastasis was remarkably decreased, indicating that the enhanced metastasis promoted by FOXM1 was dependent on the function of EGFR (Fig 3C Left). It can be observed from the statistical chart that FOXM1 is essential in promoting the pathway of tumor proliferation, so FOXM1 needs to rely on EGFR as a bridge to perform the function of tumor formation. If EGFR is inhibited, FOXM1's ability to promote cell migration will be relatively weakened (Fig 3C Right). In conclusion, we concluded that the tumorigenicity of FOXM1 was closely related to the up-regulation of EGFR.

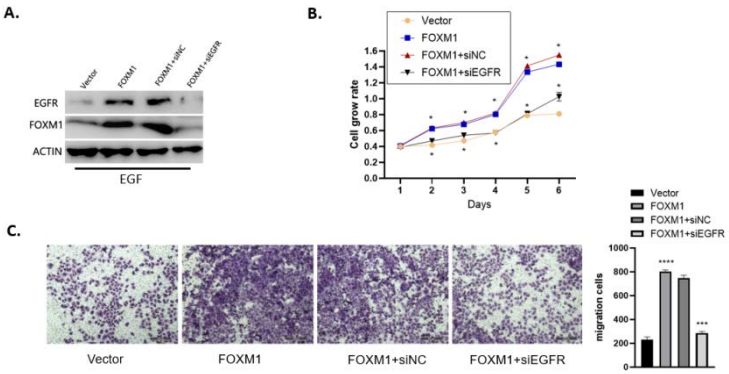

Fig.3 The Tumorigenicity of Foxm1 is Related to the Up-Regulation of Egfr

A.Western Blot detected FOXM1 and EGFR expression in FOXM1 overexpression group and knockdown EGFR group.

B.CCK-8 assay analyzed the cell proliferation ability in FOXM1 overexpression group and knockdown EGFR group.

C.Migration assay was perform to verify the motility in FOXM1 overexpression group and knockdown EGFR group.

4.4 Foxm1 Activates the Phosphorylation of Proteins in the Downstream Signaling Pathway of Egfr

Western Blot results showed that after FOXM1 overexpression, expression of EGFR increased, expression of phosphorylated EGFR, MEK, and ERK also increased (P-EGFR, P-MEK, p-ERK). Phosphorylated proteins represented the activated state, so MEK and ERK needed to be activated by phosphorylated EGFR to produce downward signal transduction and function. When FOXM1 is overexpressed and EGFR is suppressed, the downstream signaling pathway of EGFR will be unable to receive the signaling signal of EGFR so that these downstream signaling pathway proteins will not be activated, resulting in the suppression of the tumorigenesis effect of FOXM1. Thus, FOXM1 activates EGFR-associated downstream signaling pathways and thus performs tumor promotion.

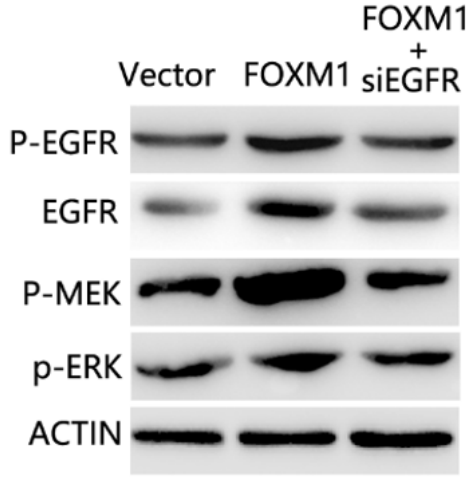

Fig.3 Foxm1 Activates the Phosphorylation of Proteins in the Downstream Signaling Pathway of Egfr

5. Discussion

It has been confirmed that FOXM1 is highly expressed in varieties of cancers, such as lung cancer [9], glioblastoma [10], basal cell carcinoma [11], invasive ductal carcinoma [12], and intrahepatic cholangiocarcinoma [13]. Although research on FOXM1 in certain cancers, especially lung cancer, has made great progress, its role in lung cancer resistance research still needs to be deeply understood, because many lung cancer patients still cannot avoid recurrence occurred after

receiving EGFR receptor blockers treatment [8]. Overcoming the limitations of these therapies requires a better understanding of the underlying mechanisms of lung cancer chemotherapy resistance recurrence. Most of the research on the factors that determine the response of cancer patients to EGFR receptor blockers treatment focuses on the intrinsic mutations of the tumor, such as EGFR mutations, rather than the expression of FOXM1 and EGFR in tumor cells. The research to determine these new mechanisms will promote the establishment of new treatment strategies.

To our knowledge, this study is the first to corroborate that EGFR has been always regulated by FOXM1 at the transcriptional level. Overexpression of FOXM1 promotes the expression level of EGFR. Further studies have shown that EGFR plays an indispensable role in the division and growth of tumor cells induced by FOXM1. Moreover, we have shown that while FOXM1 promotes EGFR expression, it also activates the downstream MEK signaling pathway of EGFR.

EGFR is the receptor protein of EGF, the family member of the ErbB/HER family. It cannot be ignored that the ErbB/HER family also contains members ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4, regulating an intricate system of signaling network that affects varieties of cellulate processes such as cell propagation, survival, and metastasis [2]. Through activating the PI3K-AKT and RAS-RAF-MEK-MAPK pathways, the expression levels of EGF and EGFR could be detected to increase dramatically, thus the migration of tumor cells will increase relatively. To accurately differentiate normal cells and tumor cells, epidemiologists identify growth factor independence to obtain satisfactory results. As mentioned above, due to the independence of cell growth factor, it means that cancer cells do not require external signal stimulation in the form of growth factors to proliferate. In ontogenesis, both EGFR and its family members are actively involved and overexpressed. On account of the frequent overexpression of EGFR and FOXM1 in a variety type of human cancers, we first demonstrate that the expression of EGFR is regulated by FOXM1. Because the downstream signal pathway of EGFR is essential for the occurrence and development of tumors, and FOXM1 can promote the transduction of its downstream signals through EGFR, which puts cells in an environment stimulated by intense growth signals, and finally makes tumor cells growth frantically and infinitely.

References

[1] Fred R Hirsch, Giorgio V Scagliotti, James L Mulshine, Regina Kwon, Walter J Curran, Yi-Long Wu, Luis Paz-Ares. Lung cancer: current therapies and new targeted treatments

[2] Stephan Lindsey, Sigrid A. Langhans. Epidermal Growth Factor Signaling in Transformed Cells.

[3] Fenghua Zeng, Raymond C. Harris. Epidermal growth factor, from gene organization to bedside.

[4] Sara Sigismund, Daniele Avanzato, Letizia Lanzetti. Emerging functions of the EGFR in cancer.

[5] Laoukili J, Stahl M, Medema RH. FoxM1: At the crossroads of ageing and cancer. Biochim Biophys Acta 2007;1775:92–102.

[6] Pilarsky C, Wenzig M, Specht T, Saeger HD, Grutzmann R. Identification and validation of commonly overexpressed genes in solid tumors by comparison of microarray data. Neoplasia 2004;6:744–50.

[7] Halasi M, Gartel AL. FOX(M1) News–It Is Cancer. Mol Cancer Ther 2013;12:245–54.

[8] Gilda da Cunha Santos,Frances A Shepherd,Ming Sound Tsao. EGFR mutations and lung cancer. Annu Rev Pathol. 2011;6:49-69.

[9] Mitsuuchi, Y. et al. Cytogenetics and molecular genetics of lung cancer. Am. J. Med. Genet. 115, 183–188 (2002).

[10] Van den Boom, J. et al. Characterization of gene expression profiles associated with glioma progression using oligonucleotide-based microarray analysis and real-time reverse transcription-polymerase chain reaction. Am. J. Pathol. 163, 1033–1043 (2003).

[11] Teh, M. T. et al. FOXM1 is a downstream target of Gli1 in basal cell carcinomas. Cancer Res. 62, 4773–4780 (2002).

[12] Wonsey, D. R. & Follettie, M. T. Loss of the forkhead transcription factor FoxM1 causes centrosome amplification and mitotic catastrophe. Cancer Res. 65, 5181–5189 (2005).

[13] Obama, K. et al. Genome-wide analysis of gene expression in human intrahepatic cholangiocarcinoma. Hepatology 41, 1339–1348 (2005).