Profilng analysis of FOX gene family members identified FOXE1 as potential regulator of NSCLC development

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Abstract: Lung cancer is one of the most malignant tumors worldwide with a high mortality rate, which has not been improved since several decades ago. FOX gene family members have been reported to play extensive roles in regulating many biological processes and disorders. In order to clarify the contribution of FOX gene family members in lung cancer biology, we performed expression profiling analysis of FOX gene family members from FOXA to FOXR in lung cancer cell lines and tissue specimens by Real-time PCR, western blot and immunohistochemistry analysis. We found that FOXE1 was the only gene which was over-expressed in six out of eight lung cancer cell lines and human cancer tissue specimens (28 out of 35 cases with higher expression and 7 out of 35 cases with moderate expression). Further investigation showed that MMP2 gene was up-regulated, and autophagy markers such as LC3B, ATG5, ATG12 and BECLIN1, were down-regulated concomitant with the increase of FOXE1. These results implicated that FOXE1 may be an important regulator by targeting autophagy and MMPs pathways in lung cancer development.

Key words: FOXE1, Non-small-cell lung carcinoma, autophagy, MMPs pathway.

Introduction

Lung cancer is the most lethal malignant neoplasia, which causes 30% of all cancer-related deaths in both sexes throughout the world (1). As the major type of epithelial lung cancer, Non-small-cell lung carcinoma (NSCLC) accounts for about 85% of all lung cancers. Due to lack of effective screening strategies, almost three fourths of lung cancers were first diagnosed at advanced stage (stage III or IV), leading to its high five-year mortality rate. Compared with other cancers such as breast cancer, of which the survival has been significantly improved, no advance has been made to ameliorate the mortality rate for lung cancer during the past several decades (2).

The malignant transformation of lung cancer was regarded as the outcome of multistep evolution by the accumulation of genetic and epigenetic aberrations, which conferred invasion, metastasis and chemo-resistance to the cancer cells during clonal expansion (3). Genetic aberrations may vary including point mutations, rearrangement and somatic copy number alterations, etc (4). Mutations in genes that encoding kinases and tumor-associated genes are commonly identified to be critical events in lung cancer initiation and development (5). For example, activating mutations in many proto-oncogenes including KRAS, EGFR, BRAF, PI3K, MEK and HER2 played essential roles in regulating proliferation, apoptosis, and any other malignant phenotypes in lung cancer progression. Meanwhile, other genetic or epigenetic alterations were all major contributors of lung cancer development, such as: structural rearrangements in ALK, ROS1; amplification of proto-oncogenes as MET, FGFR1 and DDR2; and epigenetic regulation by microRNAs (miRNAs); inactivation of Tumor Suppressor Genes, etc (6).

The forkhead (FOX) gene family is composed of a group of transcription factors that possess the same DNA binding domain of three alpha helices flanked by two ‘wings’ of beta strands and loops (7). Currently, over 100 FOX gene family members named FOXA to Q, which belong to 17 subclasses, have been identified to have important functions in multiple biological processes and disorders including cancer (7,8).

The relationship of FOX genes with cancer was first discovered by Vogt who found the structural resemblance between oncogene avian sarcoma virus 31 and mammalian FOXG1 gene by sharing a forkhead-type winged-helix domain (9). Meanwhile, Barr found that FOX1 gene fused with PAX3 was involved in alveolar rhabdomyosarcoma (10). FOXM1 expression is reported to be upregulated in many human cancers, and promote cancer proliferation and correlated with poor prognosis by activating cyclins, cyclin-dependent kinases and anti-apoptotic genes (11,12). Apart from these, FOXO genes were also found to be essential regulators in cancer via activation of the PI3K and Akt signaling pathways. The association of FOXO genes with cancer was further validated by genetic engineered mouse models, which showed down-regulation of FOXO1, FOXO3 and FOXO4 resulted in increased tumor susceptibility and the development of thymic lymphomas
and haemangiomas (13). FOX genes may also have dual functions in cancer development such as FOXF1, which is suggested to be a potential tumor suppressor epigenetically silenced in breast cancer. On the contrary, forced expression of FOXF1 enhanced tumorigenicity in breast carcinoma xenografts in nude mice (14). FOXF1 can also promote lung cancer cell migration by stimulating the production of hepatocyte growth factor and fibroblast growth factor-2 (15). Till now, researchers have found that FOX genes played critical roles in a large number of cancers, either as an oncogene or tumor suppressor, which was determined by the family member itself and cancer cell type (16).

Multiple FOX genes including FOXA1, FOXA2, FOXF1, FOXF2, and FOXJ1 have been implicated to regulate epithelial cell specific gene expression in lung cells (7). However, only FOXO1 and FOXF1 genes were hypothesized to be involved in lung cancer initiation and development (17,18). In order to elucidate the function of FOX genes in lung cancer pathogenesis, in this study we profiled the FOX genes family members in lung cancer patient samples by Q-PCR and immunohistochemistry, which demonstrated that FOXE1 could be the potential regulators in lung cancer development.

Materials and Methods

Cell culture and tissue specimen collection

Human lung adenocarcinoma cancer cell lines were purchased from ATCC (Manassas, USA), and cultured in RPMI media with 10% fetal bovine serum (Gibco, CA, USA). Human bronchial epithelial (HBE) cells were maintained in high-glucose DMEM medium with 10% fetal bovine serum. All cells were incubated at 37˚C in 5% CO2, with penicillin (100 U/ml) and streptomycin (100 μg/ml). Most cancer cells lines are non-small cell lung carcinoma xenografts in nude mice (14). FOXF1 can also promote lung cancer cell migration by stimulating the production of hepatocyte growth factor and fibroblast growth factor-2 (15).

RNA extraction and real time PCR

Total RNAs were extracted with Ambion RNA isolation kit (PureLink RNA mini Kit, Life Technologies, NY), and the RNAs were reverse transcribed with random primers using the High Capacity RT kit (Life Technologies, NY). Real time PCR was used to quantify mRNA expression with SYBR® Green mastermix kit (Life Technologies, NY) where β-actin was used as the internal control. Gene specific primers sequence was shown in Table 1.

Western blotting analysis and immunohistochemistry staining

Cells were lysed with 200μl lysis buffer (Cell signaling, MA, US) with protease inhibitor (Aprotinin 5 mg/ml; Leupeptin 1 mg/ml; PMSF, 10 mM) and phosphatase inhibitors cocktail (Roche, Shanghai). Cell lysate were denaturized with LDS loading buffer (Life technology, NY) and DL-Dithiothreitol (Sigma, MO, US), and loaded on a 10% Novage Bis-tris gel (Life technology, NY, US), and then transferred to nitrocellulose membrane. The membrane was incubated with anti-FOXE1 (1:1,000; anti-rabbit; ab134129, Abcam, MA, US) and anti-β-actin (1:30,000, anti-mouse; Sigma A2288, MO, US) antibody and detected by enhanced chemiluminescent (ECL) plus reagent kit or SuperSignal West Pico Chemiluminescent Substrate kit (Fisher Thermo Scientific, PA, US). Human cancer tissue arrays were deparaffinized and dehydrated with gradient ethanol, and processed with 30% H2O2-methonal solution for 30 mins for antigen retrieval. Tissue array slide was incubated with anti-FOX1 antibody (1:1,000; anti-rabbit; ab134129, Abcam, MA, US) at 4 degree over night. HRP-conjugated secondary antibody were stained at room temperature for 1 hour. Immunohistochemistry image were acquired with DAB substrate (ab64238, Abcam, MA, US).

Evaluation of immunohistochemistry

The immunohistochemistry results of each specimen were analyzed and quantified by Image J software. The

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
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<td>TGCGAGTACAGCTGATCG</td>
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<tr>
<td>FOXC2</td>
<td>CTCCTCGGTATCTCACCACCA</td>
<td>CAGGGTCGATTTCAATCACC</td>
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<td>GGACACAAGGATCTATCC</td>
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<td>FOXF1</td>
<td>CGGGCTTCCGGAAGGAAATG</td>
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expression was categorized 0, +, ++, +++ and ++++ by signal intensity, which indicated the positive staining area of 0%, 25%, 50%, 75% and 100%.

Statistics
Data were shown as mean ± sd. Unpaired 2-tailed Student’s t-test was used for comparisons between groups. Fisher’s test was used to analyze the difference of FOXE1 expression between cancer and normal tissues in immunohistochemistry analysis. The p-values less than 0.05 were considered as statistically significant.

Results
In order to elucidate which FOX family member could be regulator of lung cancer, we performed expression profile for multiple FOX family members in eight lung adenocarcinoma cell lines and eleven pairs of lung cancer tissue specimens. These FOX genes include FOXA1, FOXC1, FOXC2, FOXE1, FOXF1, FOXG1, FOXL1, FOXL2, FOXM1, FOXN3, FOXO1, FOXO3, FOXO4, FOXR1, FOXP1 and FOXP3. Expression profiling revealed several deregulated FOX genes in lung adenocarcinoma cells as shown in Figure 1. FOXC2, FOXM1, FOXN3 and FOXO3 were down-regulated in majority of lung cancer cells compared with HBE (normal bronchia epithelial cells) (p<0.05) (Figure 1I, J & L). FOXE1 and FOXO1 were up-regulated in lung cancer cells compared with control (Figure 1D & K).

Meanwhile, in tissue specimens, the expression level of FOXC2, FOXE1, FOXN3, FOXO1, FOXO3 and FOXO4 were elevated in lung cancer tissues compared with normal tissue (p<0.05) (Figure 2C-H, J-M). No other alteration was found for other FOX gene members accordingly. These results revealed the expression profile of FOX gene members in lung cancer. Summarization of the data from cell lines and tissue specimens of lung cancer suggested that FOXE1 and FOXO1 might be an important factor in lung cancer development.

To validate this result, we examined the protein expression of FOX gene members in lung cancer tissues and cell lines. We found that FOXE1 showed increased expression in lung cancer cell lines by western blot analysis (Figure 3C). Immunohistochemistry results in 35 lung cancer patients and 29 normal tissues also showed the significantly higher expression of FOXE1 in lung cancer tissues (p=0.001, Figure 3A). The ratio of high expression of FOXE1 protein (which is defined as signal intensity ++ to ++++) in lung cancer tissues is 75.7%, which is only 25.9% in normal tissues. Whereas low expression of FOXE1 (0 to +) was mostly found in normal tissues with ratio of 74.1%, which is only 24.3% in tumor samples (Figure 3B). These results demonstrated that FOXE1 could be a potential regulator of lung cancer development.

Further we examined several genes which might be downstream genes of FOXE1 gene in lung cancer tissue specimens, such as: MMP1, MMP2, MMP3, MMP7, MMP9, MMP13, which belong to matrix metal-
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Lipoprotease family (19); GLI1, GLI2 and PTCH1 genes, which are effectors of Hedgehog pathway (20) and autophagy markers LC3B, ATG5, ATG12 and BECLIN1 were all down-regulated in tumors (Figure 4A-E). These data indicated that FOXE1 may regulate lung cancer development through autophagy and MMPs pathways.

Discussion

In this study, we found that FOXE1 was over-expressed at both mRNA and protein level in NSCLC cell lines as well as in human lung cancer specimens. This implicated that FOXE1 may play an essential role in lung cancer development.

Previous reports have reflected our hypothesis. Previously FOXE1 expression was found in eight cancer types (22), which suggested its potential significance in cancer development. What is interesting is that FOXE1 may take on dual functions in regulating cancer growth. In thyroid carcinoma, FOXE1 was reported to be a key factor which can unwind compact chromatin to promote transcriptional regulation (23). The susceptibility to thyroid cancer was also associated with FOXE1 gene SNP polymorphism (24). Additionally, FOXE1 can enhance cancer proliferation by regulating miR-442 in a feedback loop in hepatocarcinoma (25), and was found to be up-regulated by Hedgehog-GLI signaling activation in basal cell carcinoma (24). Meanwhile, Venza found that FOXE1 was down-regulated by high frequency of

![Image of Figure 2](image-url)

Expression profiling of 16 FOX gene family members in NSCLC tissue specimens by real-time PCR. β-actin was used as internal control. All Data are mean ± sd of triplicate values, p < 0.05 is considered statistically significant. Y-axis indicated the log10 ratio of relative expression level of each FOX gene member in Figure 2B, G, H, N and P.

![Image of Figure 3](image-url)

FOXE1 gene was up-regulated in paraffin embedded NSCLC tissue specimens by immunohistochemistry and western blot. A. Representative immunohistochemistry staining of FOXE1 in lung cancer tissues. Scale bar represents 100μm. B. Statistical analysis on the comparison of FOXE1 expression between lung cancer and adjacent normal tissues. All Data are mean ± sd of triplicate values, p < 0.05 is considered statistically significant. C. FOXE1 expression in lung cancer cell lines. β-actin was used as loading control.
promoter hypermethylation in squamous cell carcinoma (SCCs), which indicated that FOXE1 was an essential factor in regulating SCC (26). Gene promoter hypermethylation of FOXE1 genes was also identified in most patients with colitis-associated colorectal cancer, and was highly associated with disease severity (27).

Other FOX family members may also have important functions in lung cancer. FOXF1 was found to be expressed in cancer associated fibroblast (CAFs) of human lung cancer and is a CAF inducing factor in a hedgehog-dependent manner (17). Additionally, FOXF1 was also over-expressed and correlated with lymph node metastasis and Hedgehog pathway (28). Tamura reported that FOXF1 could play an important part in cancer cell invasion and migration as a p53 target gene (29). FOXO1 was suggested to be involved in chemoresistance mediated by paclitaxel-induced reactive oxygen species (ROS) in ovarian cancer cells (30). As was reported by Xu, acetylation of FOXO1 could inhibit non-small-cell lung cancer cell proliferation, apoptosis and tyrosine kinase inhibitor (TKI) resistance, whereas phosphorylation modification of FOXO1 exhibit the opposite functions (31). Kim reported that FOXO1 inhibits gastric cancer angiogenesis under hypoxic conditions by inactivating the HIF-1α-VEGF pathway (32). Previous reports also proposed that FOXO1 could be a favorable prognostic factor in human cervical cancer by suppressing cervical cancer growth through inhibition of cell cycle arrest and apoptosis (33).

Our results implicated that FOXE1 and FOXF1 were up-regulated in lung cancer cell lines and frozen lung cancer specimens, but not in paraffin embedding specimens. Therefore, whether FOXO1 and FOXF1 may exert their function in lung cancer needs to be further elucidated due to these limited results.

In regarding to the possible mechanism on how FOXE1 may influence lung cancer evolution, our results unraveled the deregulation of MMP2 and several key factors in autophagy pathways following the aberrant expression of FOXE1 in lung cancer tissue specimens, such as LC3B, ATG5, ATG12 and Beclin1, which have not been reported to be FOXE1 downstream targets yet. These results demonstrated that FOXE1 could play a pivotal role in the development of lung cancer via MMPs pathway and inhibition of autophagy pathways. These results implicated that MMP2 pathway and autophagy pathway might be crucial downstream effectors of lung cancer cell growth mediated by FOXE1.

Altogether, we found that FOXE1 was over-expressed in NSCLC cells and tissues, and accompanied by deregulation of autophagy and MMPs pathway factors, suggesting that FOXE1 may play an important role in NSCLC by targeting autophagy and MMPs pathways. The mechanism about how FOXE1 may influence lung cancer growth needs to be further investigated.

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**References**