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Original Research

Profiling 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 *FOXO1* 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

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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% CO_2 with penicillin (100 U/ml) and streptomycin (100 μg/ml). Most cancer cells lines are non-small cell lung cancer cells in histopathology. All adenocarcinoma cancer tissues were obtained from the second Affiliated Hospital in Harbin Medical University. All procedures were carried out with informed consent and followed the permission of Ethics Committee in Harbin Medical University.

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 200uL lysis buffer (Cell signaling, MA, US) with proteinase 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% Nupage Novex 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 deparraffined and dehydrated with gradient ethanol, and processed with 30% H_2O_2-methonal solution for 30 mins for antigen retrieval. Tissue array slide was incubated with anti-FOXE1 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 1. Gene specific primers for *FOX* gene family members.

Gene	Forward (5'-3')	Reverse (5'-3')
FOXA1	GCAATACTCGCCTTACGGCT	TACACACCTTGGTAGTACGCC
FOXC1	GGCGAGCAGAGCTACTACC	TGCGAGTACACGCTCATGG
FOXC2	CCTCCTGGTATCTCAACCACA	GAGGGTCGAGTTCTCAATCCC
FOXE1	CACGGTGGACTTCTACGGG	GGACACGAACCGATCTATCCC
FOXF1	GCGGCTTCCGAAGGAAATG	CAAGTGGCCGTTCATCATGC
FOXG1	GAGCGACGACGTGTTCATC	GCCGTTGTAACTCAAAGTGCTG
FOXL1	GCCTCGCCCATGCTGTATC	CGTTGAGCGTGACCCTCTG
FOXL2	GGTCGCACAGTCAAGGAGC	CGCGATGATGTACTGGTAGATG
FOXM1	CGTCGGCCACTGATTCTCAAA	GGCAGGGGATCTCTTAGGTTC
FOXN3	ACTCTGACATGCCCTACGATG	TCTGACTCCTCTCTTTGTCCAC
FOXO1	TCGTCATAATCTGTCCCTACACA	CGGCTTCGGCTCTTAGCAAA
FOXO3	CGGACAAACGGCTCACTCT	GGACCCGCATGAATCGACTAT
FOXO4	GGCTGCCGCGATCATAGAC	GGCTGGTTAGCGATCTCTGG
FOXR1	TCTCACCTCCCCTTAGCGG	GGACCATCCTTATCAGGGTTGG
FOXP1	TATGGCTGTGAGACACGTT	TATGGCTGTGAGACACGTT
FOXP3	GTGGCCCGGATGTGAGAAG	GGAGCCCTTGTCGGATGATG

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-

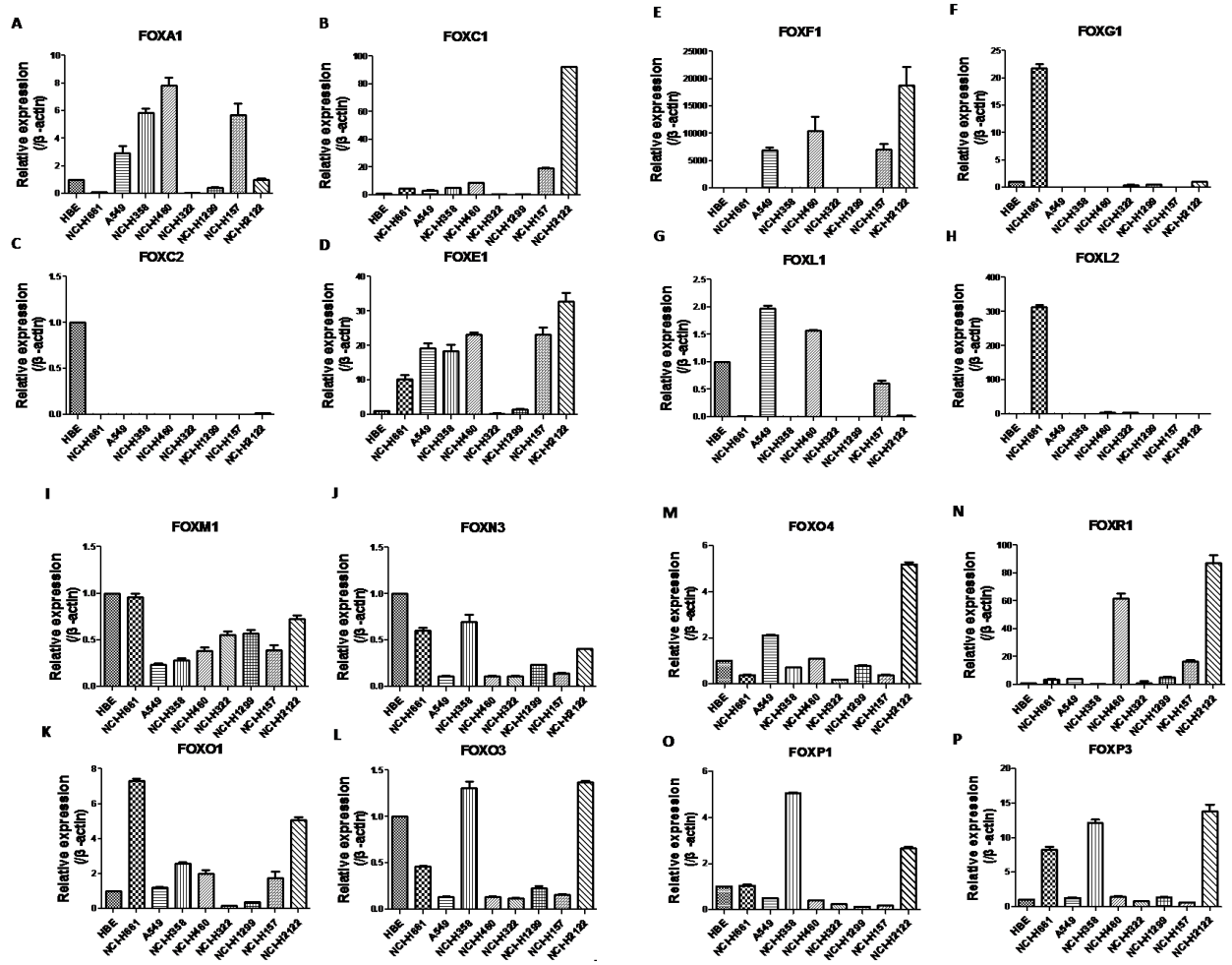


Figure 1. Expression profiling of 16 *FOX* gene family members in NSCLC cell lines by real-time PCR. β-actin was used as internal control. Data were normalized to β-actin and expressed as the mean ± sd of triplicate values.

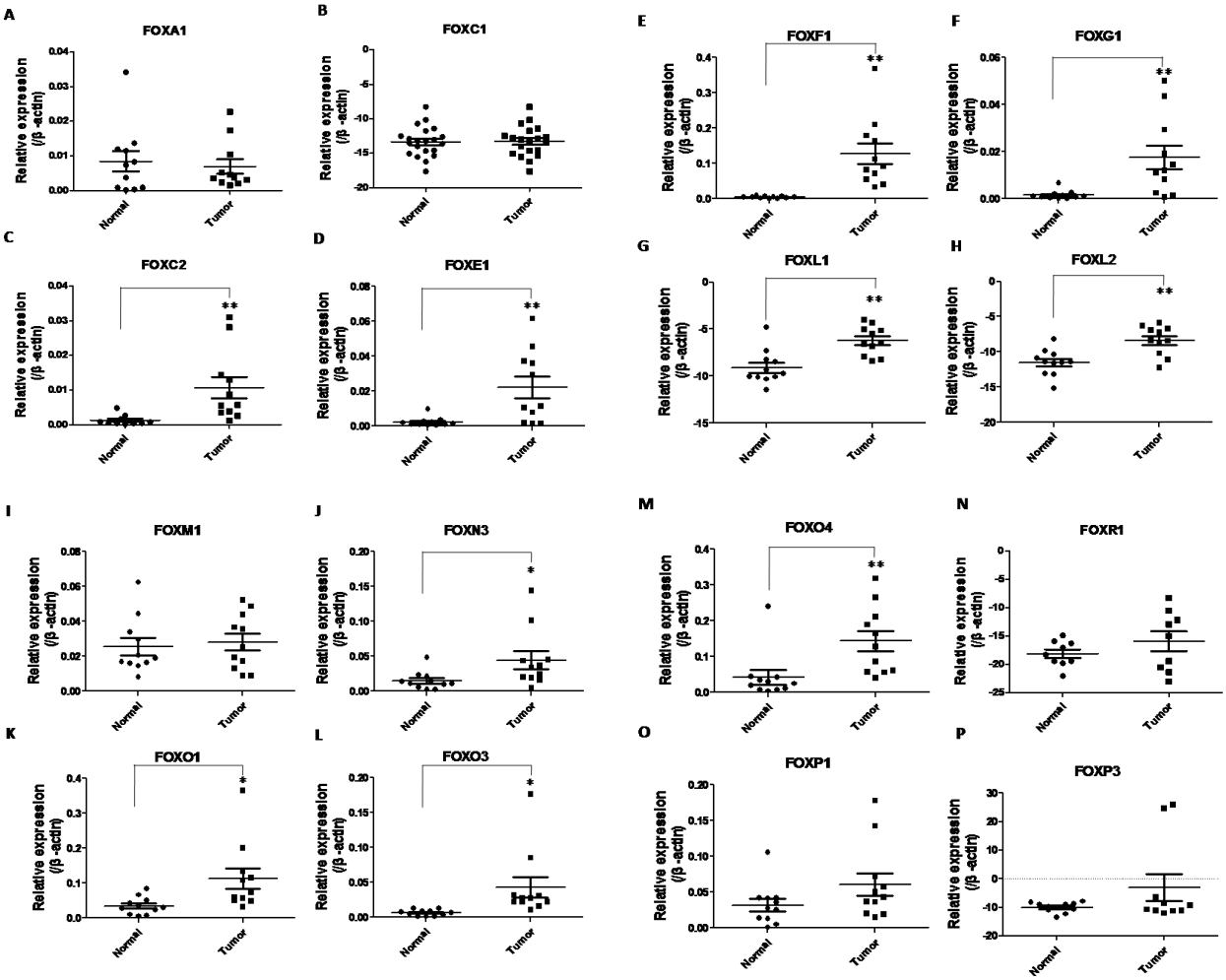
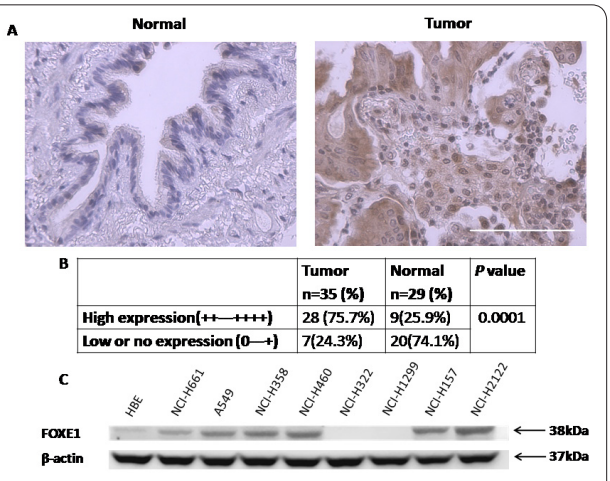


Figure 2. Expression profiling of 16 *FOX* gene family members in NSCLC tissue specimens by real-time PCR. β-actin was used as internal control. All Data are mean ± sd of triplicate values, $p < 0.05$ is considered statistically significant. Y-axis indicated the log10 ratio of relative expression level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



	Tumor n=35 (%)	Normal n=29 (%)	*P* value
High expression(++—++++)	28 (75.7%)	9(25.9%)	0.0001
Low or no expression (0—+)	7(24.3%)	20(74.1%)	

Figure 3. *FOXE1* gene was up-regulated in paraffin embedded NSCLC tissue specimens by immunohistochemistry and western blot. **A**. Representative immunohistochemisty 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.

loprotease family (19); *GLI1*, *GLI2* and *PTCH1* genes, which are effectors of Hedgehog pathway (20) and autophagy markers *LC3B*, *ATG5*, *ATG12* and *BECLIN1* (21). Results showed that *MMP2* was up-regulated in lung cancer tissues, while *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

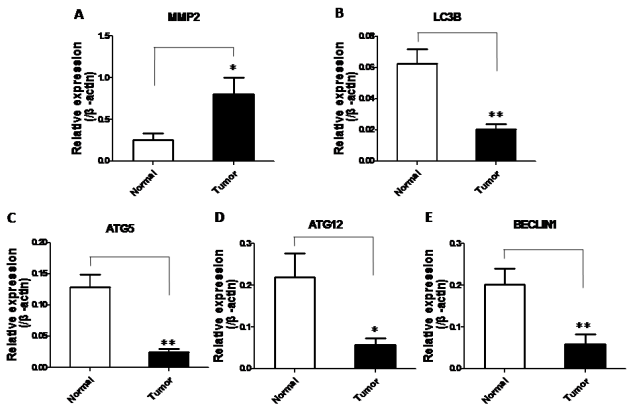


Figure 4. Autophagy and MMPs pathways markers were deregulated in NSCLC tissue specimens by real-time PCR. β-actin was used as internal control. All Data are mean ± sd of triplicate values, $p < 0.05$ is considered statistically significant.

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 chemo-resistance 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). However, our results implicated that *FOXO1* and *FOXF1* were only 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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