

Over-expression of MEOX2 promotes apoptosis through inhibiting the PI3K/Akt pathway in laryngeal cancer cells

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Early-stage diagnosis and treatment for the recurrence of carcinoma of the larynx requires further investigation. Mesenchyme homeobox 2 (MEOX2) was speculated as a novel suppressor gene in laryngeal carcinoma and the molecular mechanism was studied. Real-time quantitative PCR (RT-qPCR) and Western blot detected MEOX2 mRNA and protein levels in laryngeal cancer tissues and cells (Hep-2, TU212, AMC-NH-8 and TU686 cells), and also apoptosis and phosphoinositide 3-kinase (PI3K)/protein kinase (Akt) related factors in TU212 cells transfected with MEOX2. Cell counting kit-8 (CCK8) assay and Annexin-V/PI staining assay respectively determined the cell viability and apoptosis rates in the 46 laryngeal carcinoma patients in this study. The expression of MEOX2 was lower in larynx carcinoma tissues than normal tissues, and it correlated with the clinical stage, differentiated degree and survival time. The expression of MEOX2 was the lowest in those laryngeal cancer cells, so it was chosen for transfection in the following study. Over-expression of MEOX2 inhibited cell viability and promoted apoptosis of TU212 cells by increasing the expression levels of Caspase-3, and decreasing the levels of C-Myc, XIAP, PI3K p110 α , PI3K p110 β , PI3K class III and p-Akt. In summary, the expression levels of MEOX2 were inhibited in laryngeal carcinoma compared to normal laryngeal tissue and it correlated with cancer progression. Over-expression of MEOX2 in laryngeal cancer cells inhibited cell viability and promoted apoptosis by regulating apoptosis and PI3K/Akt pathway-related factors. This provides excellent evidence for MEOX2 use as a therapeutic gene in laryngeal carcinoma.

Key words: MEOX2, cell apoptosis, laryngeal cancer, PI3K/Akt pathway

Carcinoma of the larynx is one of the most common malignant tumors in otolaryngology; accounting for 8.4% of all malignant tumors (Statistics of China Medical University in 1983), and it ranks second in the head and neck epithelium-deprived primary malignant tumors, 90% of which were squamous cell carcinoma [1]. The incidence of laryngeal cancer is increasing because of increased exposure to a carcinogenic environment and the impact of different carcinogenic factors, [2]. Laryngeal cancer has significant gender differences; the incidence in men is approximately 10 times that of women [3]. Complicated treatment of surgical treatment and radiation therapy are used for patients with laryngeal cancer. While these can improve the annual survival rate, some patients died from tumor recurrence or distant metastasis [4]. Due to the lack of diagnostic techniques in the early-stages, many patients with laryngeal cancer are diagnosed in the late stages, thus missing the optimal treatment time [5]. The exact cause, pathogenesis and carcinogenesis of laryngeal

cancer are not very clear, and therefore the early diagnosis and treatment of its recurrence is unresolved. However some clinical data and basic experimental results have shown that smoking, drinking alcohol and human papilloma virus infection are the leading risk factors for laryngeal cancer incidence [6]. In addition, the role of genetic factors in the pathogenesis of laryngeal cancer is also gradually recognized. While the proto-oncogene may be related to the carcinogenesis, the mutation and inactivation of tumor suppressor genes could also be prime laryngeal cancer mechanisms [7–9].

The mesenchyme homeobox (MEOX) family has two homeodomain proteins, MEOX1 and MEOX2, with 95% sequence identity in the homeodomain region [10]. Both are needed for proper bone and muscle development in mouse embryos [11]. MEOX2 is also known as a specific growth arrest homeobox protein (Gax) and it is stimulisensitive and can be inhibited by stimuli [12]. MEOX2 can also inhibit cell proliferation and epithelial-to-mesen-

chymal transition (EMT) in vascular smooth muscle and endothelial cells [13, 14], and it is associated with drug resistance and therapy prognosis in lung cancer [15]. Whether MEOX2 is associated with larynx carcinoma has not been fully investigated.

Organisms conduct development, differentiation, metabolism and other normal physiological activities through accurate multi-level internal regulation mechanisms. Hence, cells lose correct temporal regulation of cell differentiation, proliferation, apoptosis and other processes when regulatory system disorders occur, and normal tissue morphology can then undergo malignant transformation to tumors. Phosphoinositide 3-kinase (PI3K)/protein kinase (Akt) signaling is an important pathway involved in the activation of anti-apoptotic mechanisms, glucose metabolism and protein synthesis which all promote normal organism cell growth and proliferation [16]. Abnormal activation of PI3K/Akt signaling is therefore closely associated with the progression of most cancers [17–19]. PI3K/Akt is also reported to participate in the progression of head and neck squamous cell carcinomas [20–22], and if MEOX2 has an inhibitory function in laryngeal cancer it may act through the PI3K/Akt pathway.

Therefore, we aimed to identify MEOX2 function in carcinoma of the larynx and to study molecular mechanisms in laryngeal cancer cells, especially in relation to the PI3K/Akt signaling pathway. Our results are expected to provide possible novel treatment of laryngeal carcinoma.

Patients and methods

Patients and tissues. Human laryngeal carcinoma tissue and normal tissue 2cm distant was collected from 46 male patients aged 50 to 70 years old in the Renmin Hospital of Wuhan University between May 2016 and May 2017. The patient history and pathological diagnoses accompanied the samples. All were diagnosed with squamous cell carcinomas; with 15 patients in the I/II stages and 31 in III/IV stages, and none had received radiotherapy or chemotherapy before total laryngectomy. The study was approved by the institu-

tional review board of the independent ethics committee, with informed consent from patients. RT-qPCR and Western blot analyses then compared MEOX2 expression levels in the cancerous and normal tissues.

Cell culture. Human laryngeal cancer cells, including Hep-2, TU212, AMC-NH-8 and TU686 cells were purchased from Shanghai Institute Chinese Academy of Sciences (Shanghai, China). Roswell Park Memorial Institute 1640 (RPMI 1640; Gibco, USA); then 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin and streptomycin (Invitrogen, USA) were used to culture the cells in 5% CO₂ and 50% humidity (37 °C). Cells of logarithmic phase were applied in following studies, and RT-qPCR and Western blot detected and compared MEOX2 expression in the different cell lines.

Cell transfection. TU212 cells were plated in 12-well plates at 6×10⁴ cells/well concentration and transfected with MEOX2 over-expressed recombinant plasmid (pcDNA3.1-MEOX2) or empty plasmid vector, using lipofectamine 2000 transfection reagent (Invitrogen, USA). The transfected cells are referred to as MEOX2 and NC groups respectively, and cells without treatment formed the Control group. The cell transfection efficiency on MEOX2 expression levels was then detected by RT-qPCR and Western blot assays.

Cell viability assay. Cell viability after transfection was determined by cell counting kit-8 (CCK8; Beyotime, Nantong, China) at determined times. Cells were cultured in 96-well plates at 5×10³ cells/well density for 12, 24 and 48 hours. CCK-8 reagent (20 µl) was then added and cells were cultured for a further hour. The production of soluble formazan was detected, and optical density (OD) values were measured at 450nm by microplate reader (Biotek, USA).

Cell apoptosis assay. The cell apoptosis rates after 48 hours transfection were determined by Annexin-V/PI double staining assay. Cells (1×10⁶ cells/well) were cultured in 6-well plates for 24 hours, harvested and stained with 5 µl Annexin V-allophycocyanin for 30 minutes at 4 °C in the dark. This was followed by staining with 10 µl propidium iodide (PI) (BD, USA). Apoptotic rates were analyzed by BD C6 flow cytometer (Beckton Dickinson, USA) and the early and late apoptotic cells were included in the quantification. Representative data of three independent assays are presented.

Realtime quantitative polymerase chain reaction (RT-qPCR). RT-qPCR detected mRNA expression levels in the tissues and cells. Total RNA extracted by Trizol reagent (Invitrogen, USA) was reversely transcribed to cDNA using PrimeScript RT Master Mix (Takara, Japan). The primers of MEOX2, C-Myc, Caspase-3, XIAP and GAPDH were synthesized by Invitrogen Company, and the sequences are displayed in Table 1. GAPDH was used as internal control. The program of PCR amplification included pre-denaturation at 95 °C for 15 seconds, 40 cycles of denaturation (95 °C, 5 seconds) and annealing/extension (60 °C for 35 seconds), using SYBR Premix Ex Taq (Takara, Japan) in ABI 7300 Thermocycler (Applied Biosystems, USA). The 2^{-ΔΔC_q} method

Table 1. The primer sequences applied in this study.

Name	Type	Sequence
MEOX2	Forward	CCAACGAAGAGGGCATGTTT
	Reverse	GGAAGAGTTGGAGCACAGGA
C-Myc	Forward	ATTCTCTGCTCTCCTCGACG
	Reverse	CTGTGAGGAGGTTTGCTGTG
Caspase-3	Forward	TGAGCCATGGTGAAGAAGGA
	Reverse	TCGGCCTCCACTGGTATTTT
XIAP	Forward	AGCAGTTGGAAGACACAGGA
	Reverse	GCAGGGTTCCTCGGGTATAT
GAPDH	Forward	CCATCTTCCAGGAGCGAGAT
	Reverse	TGCTGATGATCTTGAGGCTG

was applied for quantification and the relative expression was normalized to GAPDH. All experiments were performed in triplicate.

Western blot. The expression levels of proteins (MEOX2, C-Myc, Cleaved-caspase-3, XIAP, PI3K p110 α , PI3K p110 β , PI3K class III, p-Akt, Akt and GAPDH) were measured by Western blot. Extracted proteins were quantified by bicinchoninic acid assay (BCA) assay (Beyotime, Nantong, China), according to the factory's instructions. Total proteins were then separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 20 μ g protein in every lane and then transferred to polyvinylidene fluoride (PVDF) membranes (ThermoFisher, USA). The membranes were blocked with 5% non-fat dried skimmed milk for 1 hour and incubated with specific primary antibodies at 4°C overnight. The antibodies were purchased from Abcam (USA) and CST (USA) companies and GAPDH was used as loading control. Subsequently, the membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam, USA) at 37°C for 1 hour. Immunoreactive bands were then exposed to X-ray film and detected by enhanced chemiluminescence (ECL) reagents (ThermoFisher, USA). Finally, protein quantities were calculated using Quantity One software 3.0 (Bio-Rad, USA). All experiments were repeated three times. The antibodies used in the study were present as follows: rabbit anti-MEOX2 (Abcam, ab174116, 1:500), C-Myc (Abcam, ab39688, 1:1000), Cleaved-caspase-3 (Abcam, ab2302, 1:200), XIAP (Abcam, ab21278, 1:1000), PI3K p110 α (CST, 4249, 1:1000), PI3K p110 β (CST, 3011, 1:1000), PI3K class III (CST, 4263, 1:1000), p-Akt (CST, 4058, 1:1000), Akt (CST, 4685, 1:1000) and GAPDH (Abcam, ab9485, 1:2000).

Statistical analysis. Statistical analyses used SPSS 22.0 (SPSS, USA). The data from each triplicate assay is presented as mean \pm standard deviation. Equivalence statistical significance was determined by Student t-test and one-way analysis of variance (ANOVA). The categorical variable was determined by Chi-square test and survival analysis was analyzed by Kaplan-Meier assay. $P < 0.05$ was defined statistically significant, and $p < 0.01$ was defined especially significant different.

Results

MEOX2 expression levels correlated with larynx carcinoma progression. MEOX2 expression levels in laryngeal carcinoma tissues were determined by RT-qPCR and Western blot to determine MEOX2 biological function in this malignancy. The assays demonstrated that MEOX2 expression levels were significantly decreased in larynx carcinoma tissues compared to normal larynx tissues, both in mRNA and protein levels ($p < 0.05$, Figures 1 A–C). Figure 1B shows the representative images of Western blot. While no statistically difference in MEOX2 levels was found for age, clinical pathological features such as TNM stages and histological differentiated

grades of laryngeal carcinoma correlated with MEOX2 levels (Table 2). Carcinomas in the III and IV stages or well differentiated grades had lower MEOX2 levels. Finally, survival analysis showed that laryngeal carcinoma patients with higher MEOX2 levels (higher than in para-carcinoma tissue) had longer survival times than patients with lower MEOX2 levels (lower than in para-carcinoma tissue) (Figure 1D).

Over-expression of MEOX2 inhibited cell viability of laryngeal cancer cells. MEOX2 levels in Hep-2, TU212, AMC-NH-8 and TU686 laryngeal cancer cell lines were investigated to elucidate MEOX2 biological function in carcinoma of the larynx. The results suggested that MEOX2 expression levels were lower in laryngeal cancer cells than in normal laryngeal cells; with the lowest in TU212 cells which were chosen for further study ($p < 0.05$, Figures 2A, B). We then constructed a MEOX2 over-expression model by transfecting MEOX2 into TU212 cells in order to determine MEOX2 molecular mechanisms in laryngeal cancer cells. The detected MEOX2 mRNA and protein levels significantly increased in the MEOX2 group compared to NC and Control groups ($p < 0.01$, Figures 2 C, D). The cell viability of MEOX2 group was evaluated by CCK-8 assay and viability was time-dependently inhibited to 81.3% at 12 hours, 62.8% at 24 hours, and 52.6% at 48 hours compared to NC and Control groups ($p < 0.05$, Figure 2E).

Over-expression of MEOX2 inhibited laryngeal cancer cell apoptosis.

The inhibitory function of MEOX2 stimulated us to examine TU212 cell apoptosis rates 48 hours after MEOX2 transfection. The apoptosis rates were examined by Annexin-V/PI double staining assay, and results indicated that MEOX2 over-expression improved cell apoptosis rates significantly to 7-fold the apoptosis rates in NC and Control groups ($p < 0.05$, Figure 3A). We then measured the expression levels of apoptosis related factors, and RT-qPCR and Western blot demonstrated that the Cleaved-caspase-3 mRNA and protein levels were significantly increased and C-Myc and XIAP were notably decreased in the MEOX2 group compared to the NC and Control groups ($p < 0.01$, Figures 3B, 3C). This confirmed that MEOX2 over-expression promoted cell apoptosis of TU212 cells by regulating the expression of apoptosis-related factors.

Table 2. Tissue assay of MEOX2 expression in laryngeal carcinoma.

Factors		MEOX2 levels		p-values
		Lower	Higher	
Age (years)	<60	14	6	0.550
	≥ 60	16	10	
TNM stages	I, II	6	9	0.024*
	III, IV	23	8	
Histological grade	Middle differentiated	5	9	0.005**
	Well differentiated	25	7	

* $p < 0.05$, Chi-square test.

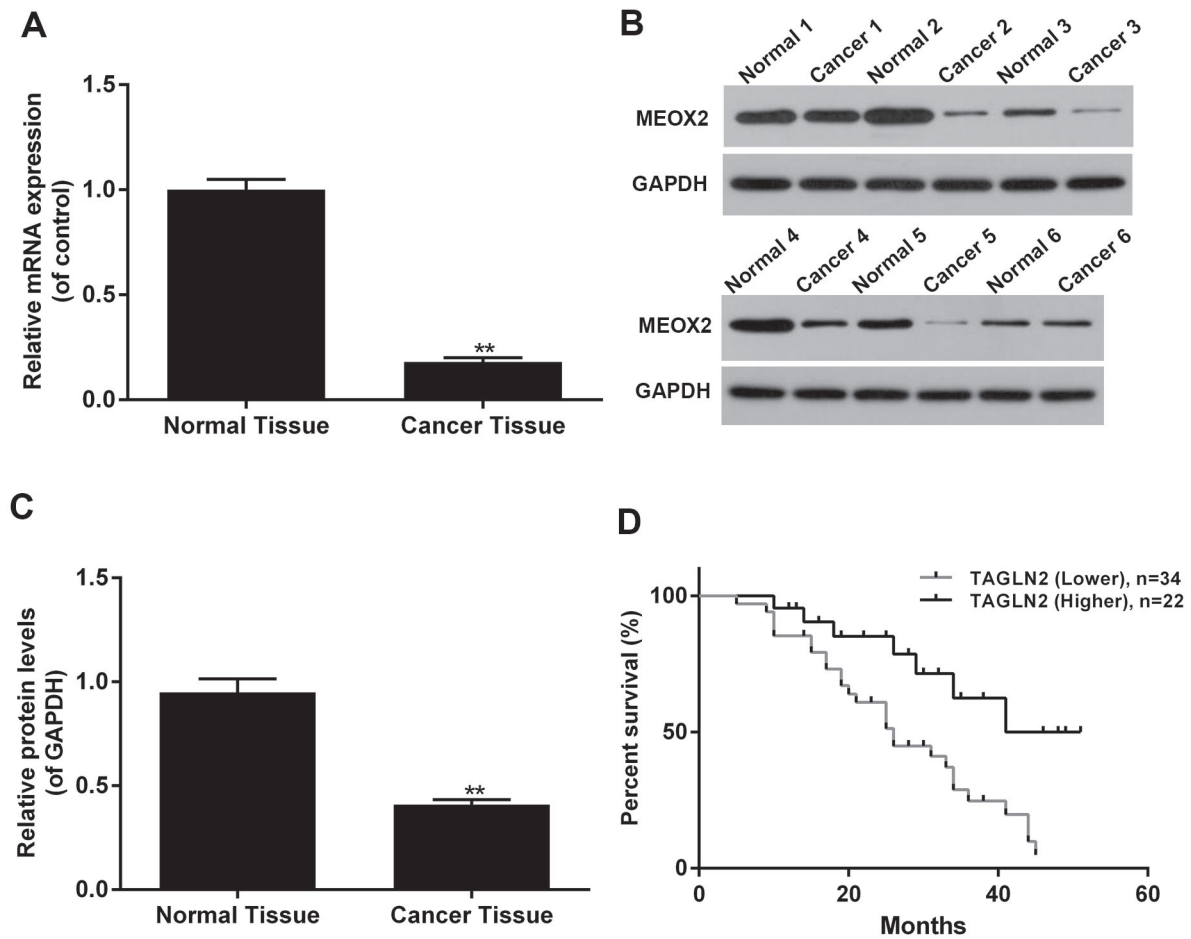


Figure 1. The expression levels of MEOX2 correlated with progression of larynx carcinoma. The mRNA and protein levels of MEOX2 in larynx carcinoma tissues were measured by RT-qPCR (A) and Western blot (B and C). B) The representative Western blot bands are presented. D) The survival time of larynx carcinoma patients and MEOX2 expression levels were analyzed. ** $p < 0.01$ vs. Normal Tissue group.

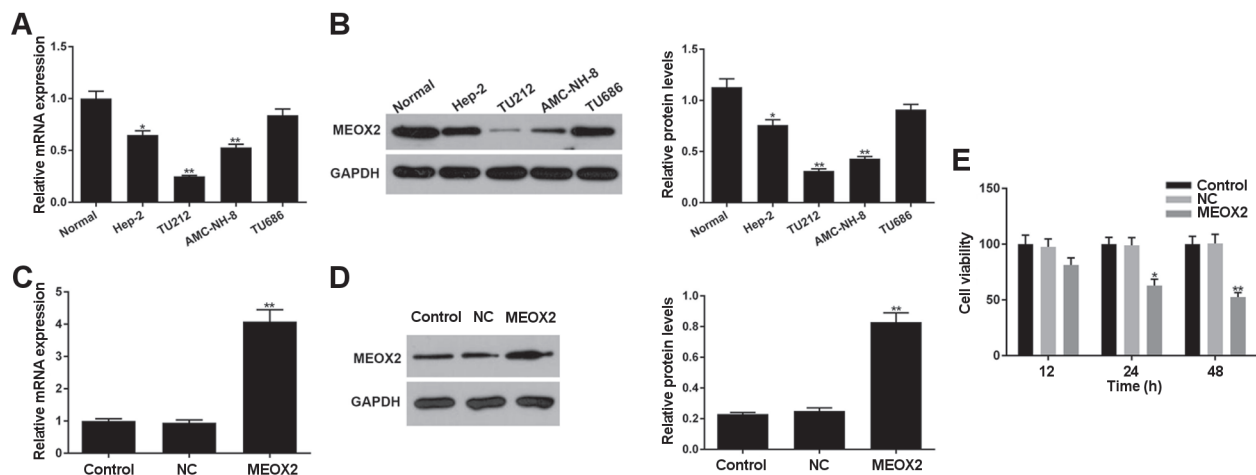


Figure 2. Over-expression of MEOX2 inhibited cell viability of laryngeal cancer cells. (A and B) The mRNA and protein levels of MEOX2 were detected by RT-qPCR (A) and Western blot (B) respectively in Hep-2, TU212, AMC-NH-8 and TU686 laryngeal cancer cells. (C and D) The mRNA and protein levels of MEOX2 were determined by RT-qPCR (C) and Western blot (D) respectively after MEOX2 transfection in TU212 cells. E) Cell viability was evaluated by CCK-8 assay at determined times (12, 24 and 48 hours). ** $p < 0.01$ vs. Normal cell group.

Over-expression of MEOX2 inhibits the PI3K/Akt pathway in laryngeal cancer cells. We next examined MEOX2's effect on the PI3K/Akt pathway because of the pathway's importance in tumor progression. Western blot was conducted to evaluate the protein levels of PI3K p110 α , PI3K p110 β , PI3K class III, p-Akt and Akt and to determine their roles in activating the PI3K/Akt pathway. The immunoblotting assay showed that these expression levels decreased significantly ($p < 0.01$) in the MEOX2 group compared with NC and Control groups ($p < 0.01$); except for little change in Akt (Figures 4A, B). The results indicated that MEOX2 promotion of apoptosis in laryngeal cancer TU212 cells was effected by PI3K/Akt pathway inhibition.

Discussion

Although genetic factors have been recognized as very important in the pathogenesis of recurrent laryngeal carcinoma, early diagnosis and treatment remains unresolved. It is therefore imperative to search for novel suppressor genes which could solve this problem. MEOX2 is reported to inhibit cell proliferation and EMT in vascular smooth muscle cells and to play an important part in lung cancer prognosis and therapy [20] [23], and it has now become urgent to investigate the molecular mechanism of MEOX2 function in carcinoma of the larynx.

Herein, we established that MEOX2 expression levels were notably lower in carcinoma tissue than in normal

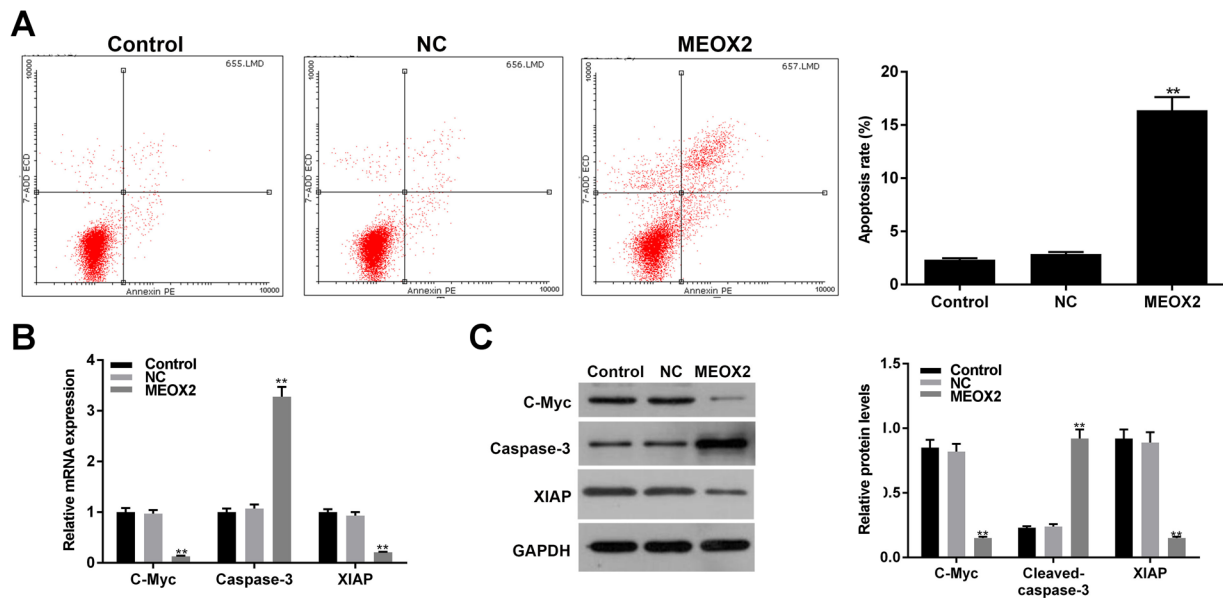


Figure 3. Over-expression of MEOX2 promoted cell apoptosis in laryngeal cancer cells. (A) Cell apoptosis were measured by Annexin-V/PI staining assay after MEOX2 transfection. (B and C) The expression levels of apoptosis related factors were examined by RT-qPCR (B) and Western blot (C) respectively. ** $p < 0.01$ vs. NC group.

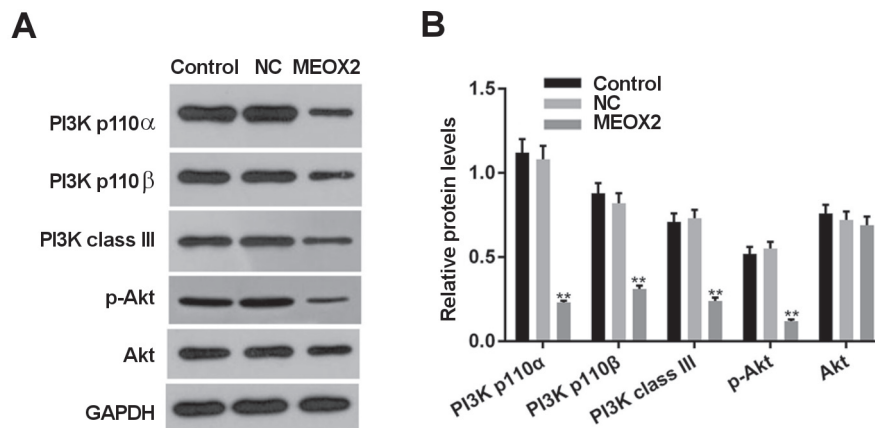


Figure 4. Over-expression of MEOX2 inhibited the PI3K/Akt pathway in laryngeal cancer cells. (A and B) The protein levels of PI3K/Akt pathway factors were determined by Western blot. ** $p < 0.01$ vs. NC group.

tissue. Moreover, much lower MEOX2 expression levels were found in the advanced stages and higher grades of laryngeal carcinoma, and patients with lower expression levels had shorter survival time. This confirms MEOX2's critical role in laryngeal carcinoma progression, so we then applied the MEOX2 over-expression technique on laryngeal cancer cells to elucidate MEOX2 functional mechanisms. The TU212 cell line was chosen for MEOX2 over-expression study because it exhibited the lowest MEOX2 laryngeal cell expression level.

Apoptosis is programmed cell death and it is essential for normal cell biological function. It is related to the activation and regulation of a series of genes conserved in species. An example is the C-Myc proto-oncogene which is the X-linked XIAP inhibitor of apoptosis protein and Caspase-3 apoptotic promotion factor [24]. Caspase-3 is called the death protease because it is a critical executive protease whose activation leads inevitably to apoptosis [25, 26]. The C-Myc transcription factor is found constitutively expressed in many cancers; including lung, stomach, breast and colon cancer and also in cervical carcinoma [27–30]. C-Myc stimulates the expression of many genes and it promotes cell proliferation and inhibits apoptosis [31] [32].

In contrast, XIAP is the strongest inhibitor of apoptosis in the IAP family and exerts its major caspase-inhibition directly [33, 34]. Over-expression of XIAP is found in most cancer cells, and it correlates with tumor progression, poor prognosis and drug resistance [35, 36]. The abnormal activation of cell proliferation and apoptosis inhibition are found in many cancers, including laryngeal cancer [37].

Hence, inhibition of cell proliferation and the activation of apoptosis provide the possibility of suppressing cancer progression. Consistent with previous research, our study demonstrated that TU212 laryngeal cancer cell viability was significantly inhibited in a time-dependent manner by MEOX2 over-expression, and cell apoptosis was dramatically promoted.

Further, RT-PCR and Western blot assays indicated that this process depended on Caspase-3 activation and C-Myc and XIAP inhibition; and this provides further evidence that the MEOX2 effect of inhibiting cell viability and promoting apoptosis inhibits laryngeal cancer progression by regulating apoptosis-related genes.

PI3K/Akt signaling is regarded as a classic anti-apoptotic pathway [38] [39]. PI3K has three classes; I, II, and III. The most widely studied is PI3K class I which can be activated by cell surface receptors [40]. PI3K class I is also divided into subtypes IA and IB which transmit signals from tyrosine kinase-coupled receptors and G-protein-coupled receptors respectively. The catalytic p110 α and p110 β IA subunits are now becoming important research tools and PI3K p110 α has been reported to be frequently mutated in many cancers including head and neck squamous cell carcinoma [41].

PI3K also directly catalyzes downstream substrates to activate the Akt signaling pathway [42, 43], and abnormal

PI3K/Akt pathway activation often exists in most cancers; especially including laryngeal cancer [22, 44].

The over-expression of MEOX2 established in our research inhibited the expression levels of the following subtypes; PI3K p110 α , PI3K p110 β , PI3K class III and p-Akt. This confirms that MEOX2 suppressed cell viability and promoted apoptosis by regulating the PI3K/Akt pathway, and the action consisted of suppressing the above five PI3K subtypes and activating Akt.

In conclusion, MEOX2 proves an effective novel cancer-suppressor because it can inhibit cell viability and promote apoptosis in laryngeal cancer cells by regulating apoptosis-related factors and the PI3K/Akt pathway. MEOX2 expression correlates with the carcinoma progression, and hence future research could well be based on *in vivo* investigation of MEOX2 treatment of this cancer. Finally, MEOX2 is a broad-prospected target gene and it could well contribute to early-stage diagnosis, gene treatment and prognosis of carcinoma of the larynx.

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