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Original Article

High expression of HMBOX1 promotes the progression of lung squamous cell carcinoma

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Abstract

This study aimed to explore the expression of homeobox-containing 1 (HMBOX1) and its clinical significance in lung squamous cell carcinoma (LSCC). Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the expression of HMBOX1 in LSCC tissues. The relationship between HMBOX1 expression and clinical pathological data was analyzed by Chi-square or Fisher's exact test. Furthermore, the role of HMBOX1 in LSCC *in vitro* was detected by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), flow cytometry and Western blot (WB) assays. HMBOX1 was highly expressed in LSCC tissues when compared with para-cancer tissues ($P<0.05$). According to the median expression of HMBOX1, the patients were divided into two groups, including high-expression group and low-expression group. HMBOX1 expression was correlated with tumor size, differentiation and clinical stage ($P<0.05$). Subsequent experiments indicated that LSCC cells with low expression of HMBOX1 exhibited significantly inhibited proliferation, G0/G1 block and promoted apoptosis ($P<0.05$). HMBOX1 expression was positively correlated with the expression of VEGF, elaborating that HMBOX1 probably promoted the growth of LSCC cells by affecting VEGF. HMBOX1 might contribute to the development of LSCC.

Keywords: Lung squamous cell carcinoma (LSCC), Homeobox containing 1 (HMBOX1), Vascular endothelial growth factor (VEGF).

1. Introduction

Lung cancer (LC) represents a significant global health concern, standing as the most common malignant tumor with widespread implications for human well-being. Its prevalence is underscored by inconspicuous clinical symptoms in the early stages and the absence of effective screening indexes and diagnostic methods. Consequently, LC holds the dubious distinction of ranking first among all malignant tumors in terms of both morbidity and mortality [1]. Approximately 13% of all new cancer cases are attributed to LC, highlighting its pervasive impact on public health. Furthermore, the overall prognosis for LC patients remains relatively bleak due to late-stage detection and the inadequacy of current treatment modalities.

Non-small cell lung cancer (NSCLC) constitutes a substantial majority of LC cases, accounting for approximately 80-85% of all instances. The 5-year survival rate for NSCLC hovers at a mere 17%, emphasizing the urgency for enhanced diagnostic and therapeutic approaches. Within NSCLC, two principal histological subtypes, namely lung adenocarcinoma (LAC) and lung squamous cell carcinoma (LSCC), stand out. LSCC, constituting over 30% of NSCLC cases, particularly prevails among smoking men [2]. While considerable progress has been achieved in comprehending the pathogenesis, developmental mechanisms, and targeted drug therapies for LC, the majority of research endeavors have been concentrated on LAC. This has led to a notable disparity in scientific exploration and clinical outcomes concerning LSCC. Consequently, there is a compelling need to redress this imbalance and initiate comprehensive research endeavors specifically focused on LSCC [3,4].

In 2006, a seminal study by Chen et al. [5] marked a pivotal moment in molecular research by isolating homeobox containing 1 (HMBOX1) from a human pancreas complementary deoxyribonucleic acid (cDNA) library. Situated between chromosome 8p12 and 8p21.1, HMBOX1 is a highly conserved gene with widespread expression in human tissues. The presence of a homeobox domain at the N-terminus categorizes HMBOX1 into the homeobox family. Notably, while most genes in this family possess a domain of 60 amino acids, HMBOX1 exhibits a longer domain with 78 amino acids [6]. The C-terminus of the HMBOX1 molecule features a hepatocyte nuclear factor-1 (HNF1)-N domain, designating HMBOX1 as part of the HNF subfamily5. Previous investigations [7,8] have unveiled abnormal expressions of molecules in the homeobox gene family in various solid tumors. These molecules

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are intricately linked to critical cellular processes such as proliferation, apoptosis, tumor metastasis, angiogenesis, and DNA repair. Consequently, delving into the homeobox family promises to offer novel theoretical foundations for preventing and treating malignancies.

Despite the wealth of knowledge on the homeobox family, the specific role of HMBOX1 in LSCC has been largely neglected in scientific inquiry. The scarcity of investigations into HMBOX1's involvement in LSCC prompts the present study to bridge this critical gap in understanding. By elucidating the precise role of HMBOX1 in LSCC, this research aims to contribute valuable insights into the molecular mechanisms underpinning the development and progression of this particular subtype of lung cancer. The intricate interplay between HMBOX1 and LSCC holds the potential to uncover novel therapeutic targets and diagnostic markers, thereby paving the way for more effective interventions and improved clinical outcomes.

2. Materials and methods

2.1. Tissue Specimens Collected

LSCC tissues and para-carcinoma tissues (more than 5 cm away from cancer tissues) were harvested from 80 patients undergoing radical or palliative resection from July 2021 to June 2023. The personal information was collected, including age, gender, smoking history, CEA expression, SCC expression, tumor size, differentiation, pleural, vascular and lymphatic invasion and clinical stage. The histological grading of LSCC conformed to the reference standards in 2015 WHO *Histological Classification of Lung Cancer*. The criteria of pathological staging for LSCC patients were in line with the 8th edition AJCC/UICC *TNM Staging System for Lung Cancer*. All pathologically diagnosed tissues were freshly resected and immediately cryopreserved in liquid nitrogen for use. No patients received chemo-radiotherapy before operation. This study was approved by the Ethics Committee of The First Affiliated Hospital of Bengbu Medical College. Signed written informed consent was obtained from all participants before the study.

2.2. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequent, extracted RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) according to the AMV reverse transcription kit (2 μg total RNA added into 20 μL system) (TaKaRa, Otsu, Shiga, Japan). Real-time PCR was conducted in a PCR instrument using the 2×SYBR Green PCR Mastermix (TaKaRa, Otsu, Shiga, Japan). Briefly, an appropriate amount of cDNA was taken as the template. Next, the corresponding forward and reverse primers were designed and synthesized according to the target gene, followed by amplification (primer concentration: 0.4 μmol/L, 15 μL system). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. Three parallel samples were set for each sample. Primers used in this study were as follows: HMBOX1: forward primer: 5'-GGCAACAGCGATCMGGAGA-3', reverse primer: 5'-ATGCACCAAGCACAGAGGG-3'; GAPDH: forward primer: 5'-CGCTCTCTGCTCCTCCTGTTC-3', reverse primer: 5'-ATCCGTTGACTCCGACCTTCAC-3'. After three independent experiments, the data were analyzed using $RQ=2^{-\Delta\Delta Ct}$.

2.3. Cell Culture and Transfection

SK-MES-1 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 100 mL/L fetal bovine serum (FBS; HyClone, South Logan, UT, USA), 100 U/mL penicillin and 100 μg/mL streptomycin, and maintained in an incubator with 50 mL/L CO_2 and saturated humidity at 37°C. Cell passage was performed once every 2-3 d. Cells in logarithmic phase with good growth status were taken for transfection. For cell transfection, the cells were first planted into 6-well plates. The medium in the well plate was replaced with 2 mL of opti-MEM medium. Next, a mixture containing Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) and si-NC RNA/si-HMBOX1 RNA was added to the plate according to manufacturer's instructions.

2.4. Cell Proliferation

SK-MES-1 cells in the logarithmic growth phase were prepared into single-cell suspension. Cell suspension was then seeded into 96-well plates at a density of 5×10^3 cells/well, followed by incubation in an incubator with 50 mL/L CO_2 at 37°C overnight. After transfection, the cells were cultured continuously. 10 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (5 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) was added at 24, 48, 72, 96 and 120 h, respectively, followed by incubation for 4 h. Next, the supernatant was aspirated, and 150 μL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was added into each well, followed by low-speed shaking for 10 min on an oscillator. Absorbance at the wavelength of 570 nm was finally detected using an enzyme-linked immunometric meter (eBioscience, San Diego, CA, USA).

2.5. Cell Cycle

Transfected cells in two groups were first made into single-cell suspension, and the density was adjusted to 1×10^9/L. Then the cells were re-suspended in pre-cooled phosphate-buffered saline (PBS) and centrifuged. After discarding the supernatant, the cells were fixed with 1.2 mL of ice-cold absolute alcohol at 4°C overnight. After centrifugation at room temperature and discarding the supernatant, the cells were re-suspended in 1 mL of pre-cooled PBS, centrifuged and collected. Next, 150 μL of propidium iodide (PI) working solution was added, and the cells were stained at 4°C in dark for 30 min. Finally, the cells in each cycle were analyzed *via* a flow cytometer (FACSCalibur; BD Biosciences, Detroit, MI, USA).

2.6. Cell Apoptosis

After transfection, the cells were collected and inoculated into a culture flask (1.0×10^5/mL) overnight. 24 h later, the cells were washed twice in PBS at 4°C, trypsinized and harvested, followed by centrifugation at room temperature. Then the cells were re-suspended with PBS at 4°C twice. After the supernatant was aspirated, 300 μL of binding buffer was added, and oscillated for complete mixing. When the cell density was adjusted to 1×10^5/mL, 5 μL of Annexin V-FITC and 10 μL of PI staining solutions were added and mixed gently, followed by reaction for 15 min in dark at room temperature. Next, the cells were added

with 200 μL of binding buffer. Cell apoptosis was finally observed through a flow cytometer within 1 h.

2.7. Statistical Analysis

Statistical Product and Service Solutions (SPSS) 23.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Experimental data were expressed as the mean ± standard deviation (SD). Comparisons among multiple groups were performed using analysis of *t*-test. The association of HMBOX1 with clinical pathological data was analyzed by Chi-square or Fisher's exact test. $P<0.05$ was considered statistically significant.

3. Results

3.1. HMBOX1 Was Highly Expressed in LSCC Tissues

The expression of HMBOX1 in 80 LSCC tissues and para-normal tissues was analyzed by qRT-PCR. As shown in Figure 1A, HMBOX1 was highly expressed in LSCC tissues ($P<0.05$). This result was similar to Dai's results for HMBOX1 in renal clear cell carcinoma [9]. Next, we analyzed the relationship between the expression level of HMBOX1 and clinical characteristics of LSCC patients. Based on the median expression level of HMBOX1, we divided the 80 LSCC patients into two groups, including high-expression group and low-expression group. As shown in Table 1, HMBOX1 expression was associated with tumor size, differentiation and clinical stage ($P<0.05$). Combining the above two results, we thought that HMBOX1 might play a promoting role in progression of LSCC.

3.2. Inhibition of HMBOX1 Expression Interfered Malignant Behaviors of LSCC Cells

SK-MES-1 cells were chosen for our *in-vitro* study. PCR and WB experiments were conducted to measure the expression of HMBOX1 after transfection. As shown

Table 1. HMBOX1 expression and clinical features of patients with LSCC.

Clinicopathological Features	HMBOX1 level		χ^2	*P*
	High	Low		
Number	40	40		
Age				
<60	14	10	0.952	0.329
≥60	26	30		
Gender				
Male	29	32	0.7882	0.431
Female	11	8		
Smoking History				
Positive	25	31	2.143	0.143
Negative	15	9		
CEA Expression				
Normal	28	23	1.352	0.245
Elevated	12	17		
SCC Expression				
Normal	14	19	1.289	0.256
Elevated	26	21		
Tumor size				
<5	12	26	9.825	0.002**
≥5	28	14		
Tumor differentiation				
Well/moderate	14	25	6.054	0.014*
Poor	26	15		
Pleural invasion				
Positive	31	23	3.637	0.056
Negative	9	17		
Vascular invasion				
Positive	26	18	3.232	0.072
Negative	14	22		
Lymphatic invasion				
Positive	29	21	3.413	0.065
Negative	11	19		
Clinical Stage				
I–III^A	14	23	4.073	0.043*
III^B–IV	26	17		

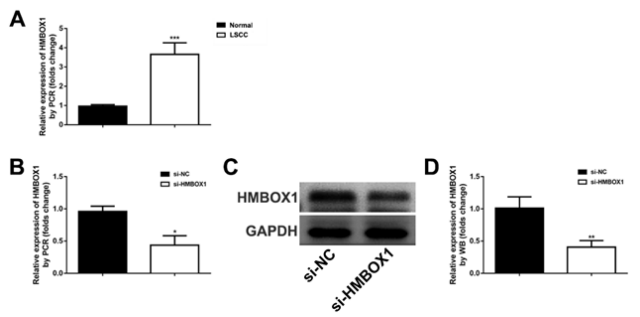

Fig. 1. (A) The expression of HMBOX1 in LSCC and para-cancer tissues was measured by qRT-PCR. (B-D) The expression of HMBOX1 in cells after transfection was measured by qRT-PCR and WB analysis. (**P*<0.05, ***P*<0.01 and ****P*<0.001).

in Figure 1B-D, both PCR and WB results confirmed the transfection efficiency. The results indicated that si-HMBOX1 transfection effectively reduced the expression of HMBOX1 in SK-MES-1 cells. Then we tested the biological activity of SK-MES-1 cells with HMBOX1 down-regulation. The results of cell proliferation (Figure 2A), cycle (Figure 2B-2C) and apoptosis (Figure 2D-2E) demonstrated that low expression of HMBOX1 could significantly change the malignant behaviors of LSCC cells. By comparing with cells treated with si-NC, si-HMBOX1 treated cells showed remarkably inhibited proliferation, G0/G1 block and promoted apoptosis (P<0.05). Based on the promoting effect of VEGF in various tumors [10-12], the protein expression level of VEGF in transfected cells was determined by WB. As the expression of HMBOX1 decreased, the expression level of VEGF decreased significantly, and they had a coordinated effect (Figure 3).

4. Discussion

LC is a major cause of cancer-related death in the world. It has been reported that the pathogenesis is correlated with tobacco, air pollution and epigenetic abnormality. Currently, the occurrence and development mechanisms as well as clinical practices related to NSCLC (the most common type of LC) have been gradually verified and applied by researchers. However, these studies are mainly focused on LAC cells represented by A549 [13-15]. Few investigations have been conducted for LSCC. Therefore, the in-depth study of the molecular mechanism of LSCC is of great significance.

Currently, the functional research of HMBOX1 is still in its infancy. It has been demonstrated that HMBOX1 conjugates with DNA target sequences by means of specific binding to helix-tum-helix (HTH) motifs containing 3 α-helices, thereby controlling cell development and differentiation. Multiple studies have also reported that HMBOX1 regulates the functions of NK cells and inhibits the lethal effect on tumor cells *via* the NKG2D/DAP10 signaling pathway [16,17]. The correlation between HMBOX1 and tumor has attracted the attention of researchers over the past few years. For example, as a cancer suppressive factor, HMBOX1 is lowly expressed in liver cancer and is involved in the regulation of multiple signaling pathways related to tumor immunity and autophagy [18]. In ovarian cancer, HMBOX1 facilitates apoptosis and represses the proliferation of cancer cells [19]. Besides, Zhou et al. [20] have discovered that silencing HMBOX1 expression in HeLa cells can promote apoptosis by decreasing the telomere length. This may eventually inhibit DNA repair and increase the radio sensitivity of HeLa cells. As a tumor promoter, however, HMBOX1 is highly expressed in renal clear cell carcinoma. In the meantime, HMBOX1 displays high expression in both carcinoma and para-carcinoma tissues of pancreatic cancer [9]. All these findings illustrate that HMBOX1 exhibits diversified expression patterns and functions in different tumors. In our study, we first detected the expression level of HMBOX1 in LSCC tissues to determine the possible role of HMBOX1 in LSCC. The results showed that HMBOX1 was highly expressed in LSCC tissues. The chi-square test indicated that HMBOX1 expression was associated with tumor size, differentiation and clinical stage of LSCC. Subsequent functional assays illustrated that down-regulation of HMBOX1 in LSCC cells significantly inhibited cell proliferation and promoted cell apoptosis *in vitro*.

Vascular endothelial growth factor (VEGF) is a key regulatory factor for tumor vascularization, which is also the most potent angiogenesis-stimulating factor known currently [21-23]. It may increase tumor vascular permeability, strengthen the invasive ability of cancer cells, and ultimately accelerate the progression, invasion and migration of malignant tumors by stimulating the proliferation and migration of vascular endothelial cells [24-26]. In our study, we found out that HMBOX1 expression was positively correlated with the expression of VEGF. These findings elaborated that HMBOX1 in LSCC cells probably promoted tumor growth by stimulating neovascularization. However, more mechanism research was still needed.

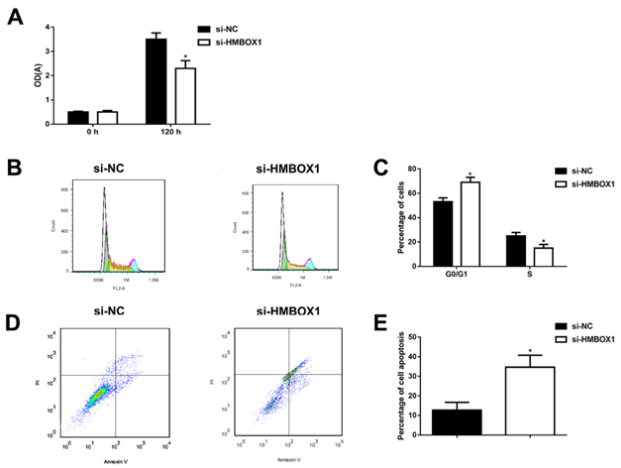

Fig. 2. (A) Cell proliferation detected by MTT assay. (B-C) Cell cycle detected by flow cytometry assay. (D-E) Cell apoptosis detected by flow cytometry assay. (**P*<0.05).

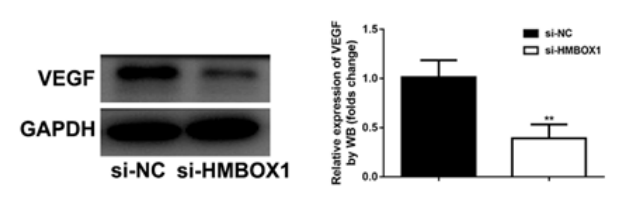

Fig. 3. The expression of VEGF in cells after transfection was measured by WB analysis. (***P*<0.01).

5. Conclusions

HMBOX1 is highly expressed in LSCC tissues and is associated with its progression. However, due to the small sample size and the lack of relevant molecular biological and laboratory data, the specific role and molecular mechanism of HMBOX1 in LSCC remains to be further studied.

Conflict of Interests

The authors declared no conflict of interest.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

This study was approved by the ethics committee of The First Affiliated Hospital of Bengbu Medical College.

Informed Consent

Signed written informed consents were obtained from the patients and/or guardians.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

XC and KW designed the study and performed the experiments, GZ collected the data, CL analyzed the data, XC and KW prepared the manuscript. All authors read and approved the final manuscript.

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