European Review for Medical and Pharmacological Sciences 2018; 22: 3067-3073

LHX6 inhibits the proliferation, invasion and migration of breast cancer cells by modulating the PI3K/Akt/mTOR signaling pathway

Q.-J. BI[1], X.-J. MEN[2], R. HAN[1], G.-L. LI[3]

[1]Department of Oncology, Fourth People's Hospital of Zibo, Zibo, Shandong, China
[2]Department of Breast Surgery, Weifang People's Hospital, Weifang, Shandong, China
[3]Department of Outpatient, Weifang People's Hospital, Weifang, Shandong, China

Abstract. – OBJECTIVE: LIM homeobox domain 6 (LHX6) is emerging as a critical regulator in carcinogenesis and tumor progression. The previous study has reported the expression and function of LHX6 in breast cancer (BC). However, its mechanism underlying BC metastasis remains largely unclear. This study aimed to investigate the related mechanisms of the tumor-suppressive role of LHX6 in BC.

PATIENTS AND METHODS: Quantitative Real-time PCR (qRT-PCR) and Western blotting were used to determine LHX6 mRNA levels and protein expressions in BC tissues and cell lines. LHX6 protein expression was also analyzed in BC tissues and matched normal breast tissues using immunohistochemistry (IHC). The biologic functions of LHX6 in BC were explored by CCK-8 assay, colony formation assay, and transwell assays in vitro. Finally, we investigated the effect of LHX6 up-regulation on PI3K/AKT/mTOR pathway by Western blot.

RESULTS: Our results showed that LHX6 was lowly expressed at the mRNA and protein level in BC cancer tissues and cell lines. Ectopic expression of LHX6 in MDA-MB-231 and T-47D suppressed cell growth, migration, and invasion. Mechanistically, our further investigations revealed that the upregulation of LHX6 inhibited the activation of the PI3K/Akt/mTOR signaling pathway.

CONCLUSIONS: We firstly provided evidence that LHX6 exerted its anti-tumor function on BC via suppressing activation of the PI3K/Akt/mTOR signaling, which eventually inhibited the progression of BC.

Key Words:
LIM homeobox domain 6, Breast cancer, Proliferation, Metastasis, PI3K/AKT/mTOR pathway.

Introduction

Breast cancer (BC) is one of the most common malignancies and the leading cause of cancer-related mortality among females worldwide[1,2]. Although great improvements have been made in the diagnosis and treatment of BC, morbidity and mortality of BC patients with advanced stages still remain high[3,4]. It is known that tumor metastasis of BC is the main reason for poor prognosis. Thus, identification of novel molecules that involve in metastasis of BC would provide insight into BC biological behaviors for potentially suppressing disease progression.

LHX genes, one of the most important subfamilies of the homeobox genes, usually have a LIM domain in addition to a homeodomain[5]. It is confirmed that LHX genes play critical roles in cellular processes such as development, proliferation, and apoptosis[6]. Emerging evidence identified LHX genes as important regulators in progression and development of various tumors[7,8]. For instance, LHX4 served as a tumor promoter in colorectal cancer by modulating the Wnt/β-catenin signaling pathway[9]. LHX9 gene, which was involved in glioma cell migration and invasiveness, was silenced in pediatric malignant astrocytomas by hypermethylation[10]. Furthermore, LHX3 and LHX5 were reported to serve as methylation biomarkers of breast cancer and neck squamous cell carcinoma[11]. As a member of the LHX family of proteins, LHX6 was found to be dysregulated in several tumors, including BC[12,13]. However, the function and potential mechanism of LHX6 in BC remains largely unclear.

In the present study, we performed RT-PCR and Western blot to detect the levels of LHX6 in both mRNA and proteins levels. Then, gain-function assay was performed to determine the function of LHX6 in BC. Finally, we performed Western blot to explore the effect of LHX6 on PI3K/AKT/mTOR pathway. Our results provided new insight into the mechanism underlying the metastasis of BC.

Corresponding Author: Guo-Li Li, MD; e-mail: ll77713@163.com

Patients and Methods

Patients and Samples

Cancerous tissues and matched non-cancerous tissue samples were collected from BC patients diagnosed by pathologists in Weifang People's Hospital between July 2016 and December 2017. No local or systemic treatment had been conducted in these patients before the operation. Tissue samples were frozen in liquid nitrogen and stored until total RNAs or proteins were extracted. Written informed consent was obtained from each individual, and the study was approved by the local Ethics Committee.

Cell Lines and Cell Culture

The human breast epithelial cell line HBL-100 and the BC cell lines MCF-7, MDA-MB-231, and T-47D were obtained from the Cell Bank of the Chinese Academy of Sciences (Xuhui, Shanghai, China). Above cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Beijing, Haidian, China), supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and 100 U/ml penicillin and 100 µg/ml streptomycin solution (Gibco, Beijing, Haidian, China) in a humidified 37°C incubator with a 5% CO_2 atmosphere.

Lentivirus Infection

LHX6 lentivirus used for LHX6 up-regulation and corresponding negative control lentivirus were purchased from GeneCopoeia, Inc. (Guandong, Guangzhou, China). One day prior to transfection, MDA-MB-231 and T-47D cells were seeded on six-well plates at a density of 3×10^5 cells per well. Lipofectamine™ 2000 reagent was used for transfections according to manufacturer's instructions. Transfection efficiency was confirmed by RT-PCR.

RNA Extraction and Reverse Transcription Quantitative PCR (RTqPCR)

Total RNA was prepared from cells using TRIzol (TaKaRa, Dalian, China). Single-stranded cDNA was synthesized by using Reverse Transcription Kit (ThermoFisher Scientific, MA, Waltham, USA). The expression levels of mRNA were determined using qRT-PCR, which was performed using the 7900 HT Sequence Detection System (Applied Biosystems, Pudong, Shanghai, China) and ABI Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The primers for LHX6 mRNA were: forward, 5'-TCAACAACCTCATCTGGCAC-3' and reverse, 5'-CATGGTGTCGTAGTGGATGC-3'. GAPDH was used as internal controls for mRNAs, respectively. The relative mRNA levels of gene expression were assessed using the $2^{-\Delta\Delta Ct}$ method.

Western Blot Assay

Cultured cells were lysed by RIPA (0.1% SDS, 1% Triton X-100, 1 mM MgCl2, 10 mM Tris-HCl, pH 7.4) at 4°C for 30 min. Thirty µg of protein lysates were separated on a NuPAGE 4-12% Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA), and the separated proteins were transferred onto a polyvinylidene difluoride membrane (Invitrogen, Carlsbad, CA, USA). The membrane was blocked with 5% skim milk for 1 h and incubated with 1:1000 diluted primary antibodies at 4°C overnight. Next, the membranes were washed and incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit) at 1:1000 dilution. Signals of the immunoreactive bands were visualized using the ECL detection system (Pierce Biotechnology, Inc., Rockford, IL, USA). GAPDH was used as a loading control.

Immunohistochemistry (IHC) Analysis

Immunohistochemistry staining was performed by using anti-LHX6 (1:600) as previously described[14]. The antigen-antibody complex was visualized by using the Metal Enhanced DAB Substrate Kit (Sigma-Aldrich, St. Louis, MO, USA).

Cell Proliferation Assay

Cells were plated at a density of 2×10^4 cells per well in 96-well microtiter plates and cultured overnight at 37°C in a humidified incubator containing 5% CO_2. At 24, 48, 72, and 96 h, 10 µl CCK-8 reagent was added to each well. Then, cells were incubated at 37°C for 4 h, and then, the absorbance at 450 nm was measured using a micro-well plate reader.

Colony Formation Assay

After lentivirus infection, MDA-MB-231 and T-47D cells were seeded in a volume of 2 mL at a density of 600 cells/well in six-well plates. Then, the plates were fixed with methanol, stained with Crystal violet solution. The number of colonies that were composed of more than 50 cells was counted in each well under a Nikon microscope.

Cell Migration and Invasion Assays

Migration and invasion assays were performed using transwell chamber inserts. 3 × 10^4 cells in 100 µl of serum-free media were added to the upper chamber of an insert coated with or without Matrigel (BD), and 1 ml DMEM containing 20% FBS was added to the bottom chamber. The cells were incubated at 37°C for a further 48 h. Non-invaded cells were removed from the upper surface of the filter carefully with a cotton swab. The stained cells were then imaged and counted with optical microscopy at 100 × magnification. All assays were independently performed three times.

Statistical Analysis

All data analyses were performed using SPSS 19.0 software (SPSS, Armonk, NY, USA). Differences between groups were analyzed using Student's *t*-test. $p < 0.05$ was considered statistically significant.

Results

LHX6 Expression is Down-Regulated in BC Cell Lines and Tissues

To explore the role of LHX6 in BC progression, we performed RT-PCR to detect the expression of LHX6 in BC cell lines and HBL-100. As shown in Figure 1A, we found that the expression level of LHX6 mRNA was downregulated in BC cell lines (MCF-7, MDA-MB-231, and T-47D) compared with HBL-100 cells. Furthermore, we also observed down-regulation of LHX6 mRNA expression in BC tissues compared to normal breast tissues (Figure 1B). Subsequently, the results of Western blot indicated that LHX6 protein levels were downregulated in BC cell lines (MCF-7, MDA-MB-231, and T-47D) compared with HBL-100 cells. Finally, IHC assay showed that LHX6 protein expression in BC tissues was usually decreased compared with that in adjacent normal tissues (Figure 1D and 1E). These results suggested that downregulation of LHX6 was involved in BC development.

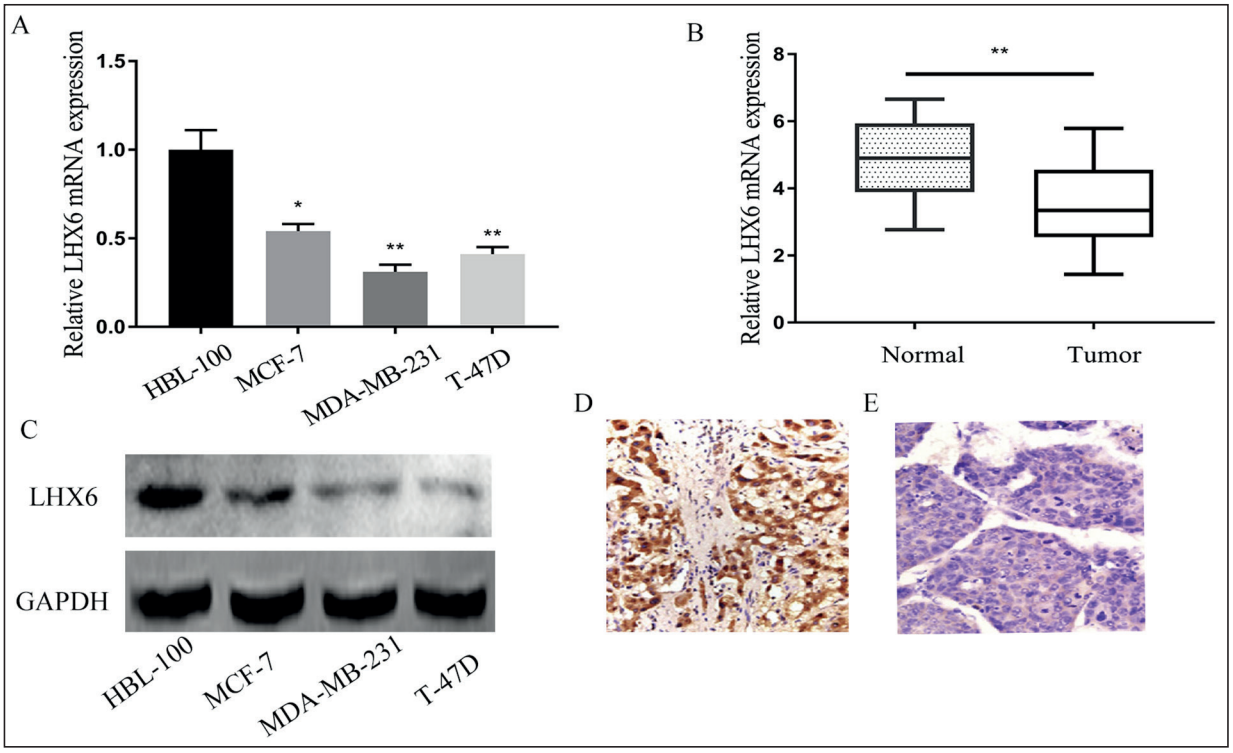

Figure 1. LHX6 expression is downregulated in both BC tissues and cell lines. **A,** The mRNA level of LHX6 was detected in BC cell lines using RT-PCR. **B,** The mRNA level of LHX6 was detected in BC tissues and normal breast tissue using RT-PCR. **C,** The protein level of LHX6 was detected in BC cell lines using Western blot analysis. **D,** High LHX6 protein expression in non-tumor tissues by Immunohistochemical analysis. **E,** Negative LHX6 protein expression in BC tissues by Immunohistochemical analysis. $^{**}p < 0.01$, $^{*}p < 0.05$.

LHX6 Suppresses Growth in MDA-MB-231 and BT-474 Cells

To investigate the biological roles of LHX6 in the growth of BC, LHX6 lentivirus and the negative control were synthesized and transfected into MDA-MB-231 and BT-474 cells. The results of RT-PCR showed that LHX6 mRNA was significantly up-regulated in cells transfected with LHX6 lentivirus (Figure 2A and 2B). CCK-8 assays revealed that overexpression of LHX6 suppressed the proliferation of MDA-MB-231 and BT-474 cells compared to NC (Figure 3C and 3D). Moreover, colony formation assay showed that the proliferative ability of MDA-MB-231 and BT-474 cells was also significantly increased after LHX6 overexpression (Figure 2E-2G). All these data demonstrated the tumor suppressive roles of LHX6 in BC cells growth.

Effect of LHX6 on MDA-MB-231 and BT-474 Cells Cell Migration and Invasion

To explore whether LHX6 affects the metastatic abilities in BC cells, we performed transwell invasion assay. As shown in Figure 3A and 3B, the experiment performed in MDA-MB-231 and BT-474 cells indicated that the number of invasive and migratory cells in the LHX6 lentivirus group was significantly lower than that in the NC group. These results provided evidence that LHX6 suppressed metastasis of BC cells *in vitro*.

Analysis of LHX6 Expression and PI3K/AKT/mTOR Pathway

It is known to us that PI3K/AKT/mTOR pathway is involved in many cellular functions, including cell cycle progression and metastasis. Thus, we wondered whether LHX6 exerted its tumor-suppressive role by affecting PI3K/AKT/mTOR pathway. The results of Western blot indicated that when MDA-MB-231 cells were transfected with LHX6 lentivirus, expressions of p-mTOR, p-P70, and p-AKT were all decreased compared with the controls (Figure 4). These results indicated that LHX6 acts as a negative regulator of the PI3K/Akt/mTOR signaling pathway.

Discussion

Tumor metastasis is a complex and multistep process, which is regulated by various molecules[15]. Several tumor-related biomarkers were identified in various tumors in the past ten years[16,17]. Those findings promote medical development enormously. In the present study, we

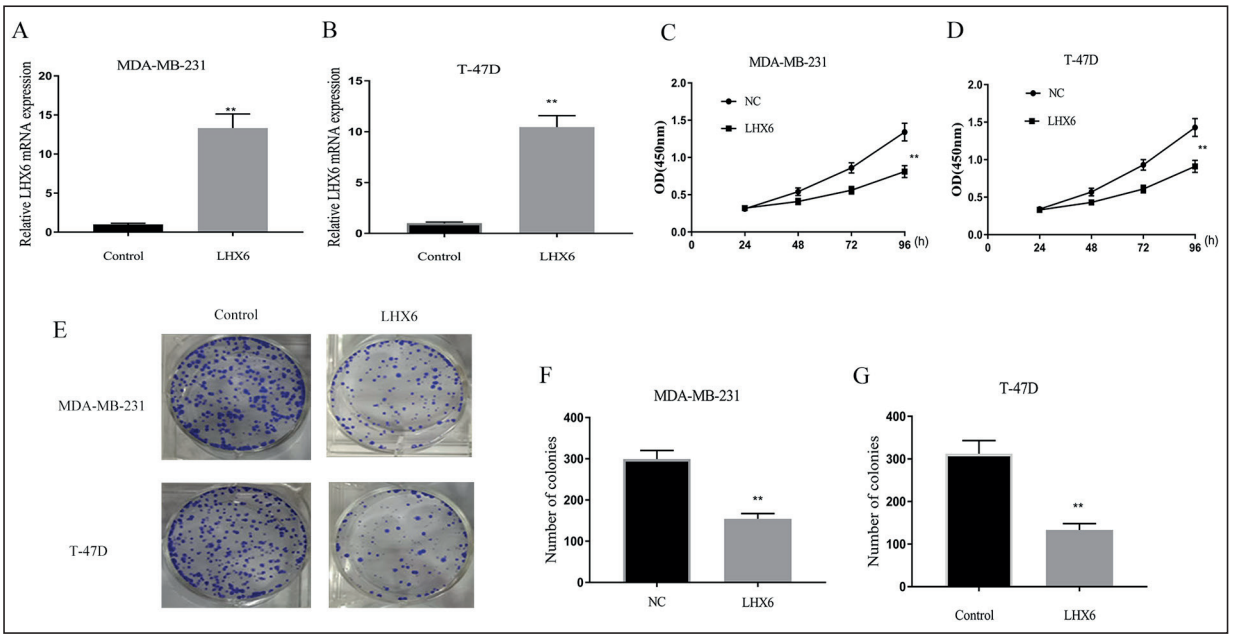

Figure 2. LHX6 upregulation inhibits BC growth and migration. **A-B,** RT-PCR detection of MDA-MB-231 and T-47D cells transfected with LHX6 lentivirus. **C-D,** MTT assay was performed to examine MDA-MB-231 and T-47D cell proliferation. **E-G,** Colony-forming assays were conducted to determine the cloning ability of MDA-MB-231 and T-47D cells, and control cells. $^{**}p < 0.01$, $^{*}p < 0.05$.

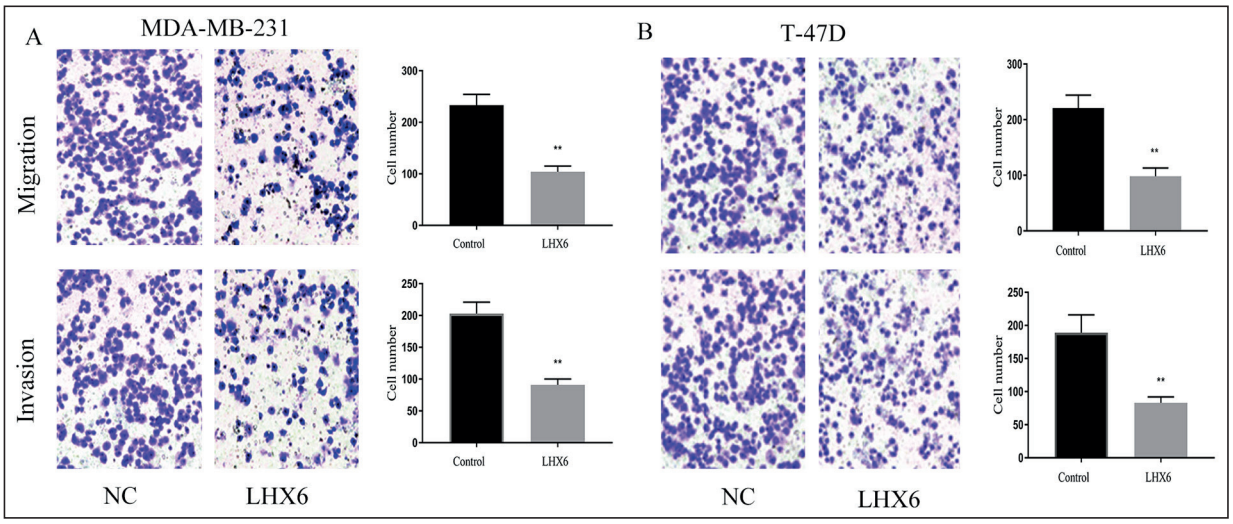

Figure 3. LHX6 upregulation inhibits the migration and invasion of BC cells *in vitro*. Transwell migration and invasion assays of MDA-MB-231 (**A**) and T-47D (**B**) cells transfected with LHX6 lentivirus or control. $^{**}p < 0.01$, $^{*}p < 0.05$.

found that LHX6 was down-regulated in human BC tissues and cell lines. Furthermore, the transfection of LHX6 lentivirus into MDA-MB-231 and T-47D cells was able to reduce cell proliferation, invasion, and migration *in vitro*. We further confirmed that overexpression of LHX6 could inhibit the activation of PI3K/AKT/mTOR pathway. To author's knowledge, this research is the first to show that the LHX6/ PI3K/AKT/mTOR pathway is involved in the invasion and migration of BC.

The biological function of LHX6 involved in the regulation of cancer progression has been reported in several studies. For instance, Yang et al[18] reported that LHX6 expression was significantly down-regulated in lung adenocarcinoma and its overexpression could suppress the proliferation and metastasis of lung adenocarcinoma cells by transcriptional silencing of β-catenin. In addition, clinical assay indicated that LHX6 was associated with lymph node status and clinical stages, as well as the overall survival of patients. Jung et al[19] reported that LHX6 could be used as an effective and sensitive methylation biomarker for early diagnosis of cervical cancer. Yan et al[20] found that LHX6, which was a target gene of miR-1290, suppressed glioma cells proliferation, migration, and invasion *in vitro* and *in vivo*. Those results highlighted the tumor-suppressive role of LHX6 in various tumors. More important, Hu et al[13] observed that the expression levels of LHX6 were down-regulated in BC tissues, and its forced expression served as a tumor suppressor by inhibiting BC cell proliferation and invasion via modulating the Wnt/β-catenin signaling pathway. In our present study, we also showed LHX6 as an anti-oncogene by up-regu-

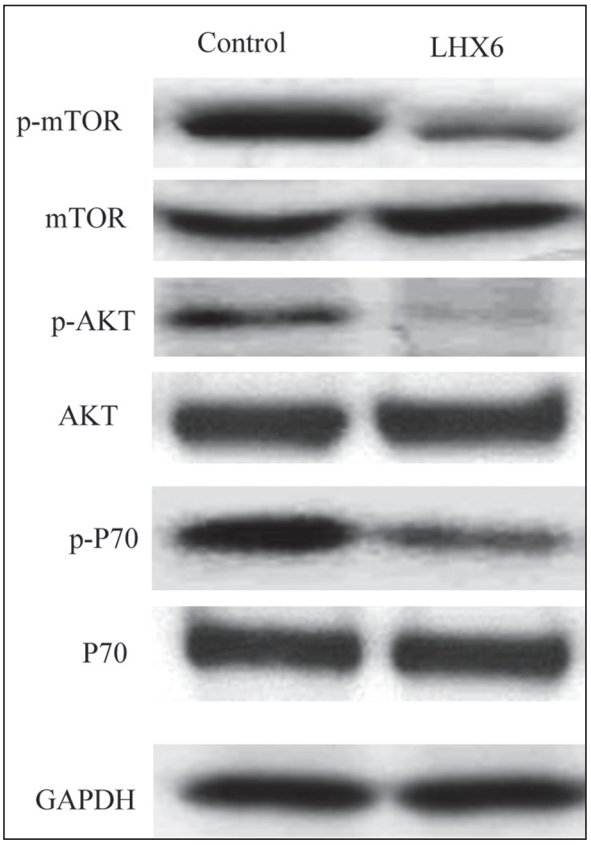

Figure 4. Influence of LHX6 overexpression on PI3K/AKT/mTOR pathway associated proteins expressions.

lating the levels of LHX6 *in vitro*. Furthermore, our attention focused on the potential mechanism by which LHX6 exerted its tumor-suppressive role in BC.

The PI3K/AKT/mTOR pathway is a signal transduction pathway, which participates in the regulation of multiple cellular progression including cell proliferation, survival, differentiation, and metastasis[21]. In recent years, the abnormal activation of PI3K/Akt/mTOR pathway has been found in various tumors, including BC, and the overactivation of the PI3K/Akt/mTOR pathway contributed to the tumorigenesis and progression[22-24]. In the present study, we performed Western blot to explore the effect of LHX6 on the PI3K/Akt/mTOR pathway, and the results indicated that up-regulation of LHX6 reduced phosphorylation levels of PI3K, Akt, and mTOR, suggesting that LHX6 served as a tumor suppressor by modulating PI3K/Akt/mTOR pathway.

Conclusions

For the first time we showed that LHX6 suppressed BC cells proliferation, migration, and invasion through the inactivation of the PI3K/Akt/mTOR pathway. LHX6 would be a potential molecular target for BC therapy.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin 2013; 63: 11-30.
2) Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011; 61: 69-90.
3) Anastasiadi Z, Lianos GD, Ignatiadou E, Harissis HV, Mitsis M. Breast cancer in young women: an overview. Updates Surg 2017; 69: 313-317.
4) Yu QT, Meng ZB. Treatment of advanced breast cancer with a combination of highly agglutinative staphylococcin and vinorelbine-based chemotherapy. Eur Rev Med Pharmacol Sci 2016; 20: 3465-3468.
5) Simmons DK, Pang K, Martindale MQ. Lim homeobox genes in the Ctenophore Mnemiopsis leidyi: the evolution of neural cell type specification. EvoDevo 2012; 3: 2.
6) Schmitt S, Biason-Lauber A, Betts D, Schoenle EJ. Genomic structure, chromosomal localization, and expression pattern of the human LIM-homeobox3 (LHX 3) gene. Biochem Biophys Res Commun 2000; 274: 49-56.
7) Zhou F, Gou S, Xiong J, Wu H, Wang C, Liu T. Oncogenicity of LHX2 in pancreatic ductal adenocarcinoma. Mol Biol Rep 2014; 41: 8163-8167.
8) Shuert C, Horning M, Mellish JA. The effect of novel research activities on long-term survival of temporarily captive steller sea lions (Eumetopias jubatus). PLoS One 2015; 10: e0141948.
9) Cha N, Liu W, Yang N, Xie S, Gao Y, Chen X, Wang X, Ren J. Oncogenicity of LHX4 in colorectal cancer through Wnt/β-catenin/TCF4 cascade. Tumour Biol 2014; 35: 10319-10324.
10) Vladimirova V, Mikeska T, Waha A, Soerensen N, Xu J, Reynolds PC, Pietsch T. Aberrant methylation and reduced expression of LHX9 in malignant gliomas of childhood. Neoplasia 2009; 11: 700-711.
11) Wang X, He C, Hu X. LIM homeobox transcription factors, a novel subfamily which plays an important role in cancer (review). Oncol Rep 2014; 31: 1975-1985.
12) Jung S, Jeong D, Kim J, Yi L, Koo K, Lee J, Kim CJ, Kim CH, An S, Yang Y, Lim JS, Kim KI, Lee MS. Epigenetic regulation of the potential tumor suppressor gene, hLHX6.1, in human cervical cancer. Int J Oncol 2011; 38: 859-869.
13) Hu Z, Xie L. LHX6 inhibits breast cancer cell proliferation and invasion via repression of the Wnt/β-catenin signaling pathway. Mol Med Rep 2015; 12: 4634-4639.
14) Li P, Sun T, Yuan Q, Pan G, Zhang J, Sun D. The expressions of NEDD9 and E-cadherin correlate with metastasis and poor prognosis in triple-negative breast cancer patients. Onco Targets Ther 2016; 9: 5751-5759.
15) Ganguly KK, Pal S, Moulik S, Chatterjee A. Integrins and metastasis. Cell Adh Migr 2013; 7: 251-261.
16) Tuo YL, Ye YF. MGP is downregulated due to promoter methylation in chemoresistant ER+ breast cancer and high MGP expression predicts better survival outcomes. Eur Rev Med Pharmacol Sci 2017; 21: 3871-3878.
17) Cheng X, Wu H, Jin ZJ, Ma D, Yuen S, Jing XQ, Shi MM, Shen BY, Peng CH, Zhao R, Qiu WH. Up-regulation of chemokine receptor CCR4 is associated with human hepatocellular carcinoma malignant behavior. Sci Rep 2017; 7: 12362.
18) Yang J, Han F, Liu W, Zhang M, Huang Y, Hao X, Jiang X, Yin L, Chen H, Cao J, Zhang H, Liu J. LHX6, An independent prognostic factor, inhibits lung adenocarcinoma progression through transcriptional silencing of β-catenin. J Cancer 2017; 8: 2561-2574.
19) Jung S, Jeong D, Kim J, Yi L, Koo K, Lee J, Lee SD, Park JW, Chang B, Kim CH, Kim CJ, Lee MS. The role of hLHX6-HMR as a methylation biomarker for early diagnosis of cervical cancer. Oncol Rep 2010; 23: 1675-1682.

20) Yan L, Sun K, Liu Y, Liang J, Cai K, Gui J. MiR-1290 promotes proliferation, migration and invasion of glioma cells by targeting LHX6. J Cell Physiol 2017 Dec 11. doi: 10.1002/jcp.26381. [Epub ahead of print].

21) Ersahin T, Tuncbag N, Cetin-Atalay R. The PI3K/AKT/mTOR interactive pathway. Mol Biosyst 2015; 11: 1946-1954.

22) Durbas M, Horwacik I, Boratyn E, Kamycka E, Rokita H. GD2 ganglioside specific antibody treatment downregulates PI3K/Akt/mTOR signaling network in human neuroblastoma cell lines. Int J Oncol 2015; 47: 1143-1159.

23) Simpson DR, Mell LK, Cohen EE. Targeting the PI3K/AKT/mTOR pathway in squamous cell carcinoma of the head and neck. Oral Oncol 2015; 51: 291-298.

24) Fu YL, Zhang QH, Wang XW, He H. Antidiabetic drug metformin mitigates ovarian cancer SKOV3 cell growth by triggering G2/M cell cycle arrest and inhibition of m-TOR/PI3K/Akt signaling pathway. Eur Rev Med Pharmacol Sci 2017; 21: 1169-1175.