

Original Article

LSD1 modulates papillary thyroid carcinoma (PTC) cell proliferation through regulating TSHR

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Abstract: In this study, we explore the roles of LSD1 in papillary thyroid carcinoma cell proliferation and investigate the mechanisms. Total of 146 patients who have been diagnosed as PTC (papillary thyroid carcinoma) were enrolled in this study. Expressions of LSD1 in PTC cell lines (IHH-4 and B-CPAP) and tissues were analyzed by qRT-PCR and western blotting. Flow cytometry was used to explore the effects of LSD1 expression on PTC cell proliferation and cell cycle. Moreover, the effects of LSD1 expression on TSHR (thyroid-stimulating hormone receptor) expression was also analyzed using ChIP (chromatin immunoprecipitation) assay. Compared to the control groups, LSD1 was significant down-regulation in PTC tissues, as well as in PTC cell lines. Silenced LSD1 dramatically promoted IHH-4 and B-CPAP cells proliferation and released cell cycle at G0/G1 stage and critical increased cyclin D1 and TSHR expression. Furthermore, LSD1 suppressed TSHR transcription via modulating H3K4 demethylation. Our findings suggested that LSD1 modulates papillary thyroid carcinoma (PTC) cell proliferation through regulating TSHR, and provided a novel biomarker to predict the prognostic.

Keywords: LSD1, cell proliferation, TSHR (Thyroid-stimulating hormone receptor)

Introduction

Thyroid carcinoma have been become the most popular endocrine malignancy [1, 2]. According to the Chinese Cancer Registry, the incidence of this cancer is 6.5 per 100,000 individuals in China [3]. The papillary thyroid carcinoma (PTC) accounts for 80% of thyroid cancers [4]. Ionizing radiation, family history and nodular disease of the thyroid are risk factors for PTC [5]. Though PTC has a relative well prognosis, but lymph node metastasis may be result in a worse oncologic outcome.

LSD1, known as KDM1, is a histone demethylase that catalyzes H3K4me via a FAD dependent oxidative reaction [6, 7]. Several evidence has been established that LSD1 plays crucial function in diverse basic cellular processes including EMT, differentiation and cell proliferation [8-12]. Furthermore, LSD1 has been reported overexpress in many human cancers, such as head and neck cancer [13-

15]. But there are also multiple studies indicated that LSD1 is down-regulate in carcinomas including breast cancer [16, 17]. However, the role of LSD1 in PTC is still unknown.

TSH (Thyroid-stimulating hormone) is a glycoprotein hormone that can specifically binding to its receptor to stimulate thyroid (thyroid-stimulating hormone receptor (TSHR) [18]. It plays a critical function in the hypothalamic-pituitary-gonadal axis to responsible for proliferation and physiological functioning of thyroid, and abnormal expression of TSHR can induce Grave's disease or thyroid cancer pathogenesis [18-21]. DNA methylation and histone modifications which have been revealed to be correlation with tumorigenesis in thyroid cancer [22]. EZH2, a histone methyltransferase which catalyzing histone H3K27me3, have been found up regulation in several tumor including thyroid cancer and influence cancer development through a diversity of regulating mechanisms [23, 24]. However, LSD1 as a histone demethyl-

transferase, the roles of it in modulating TSHR expression in papillary thyroid carcinoma have not been reported.

Materials and methods

Patients and samples

We collected 146 patients who had been diagnosed as having papillary thyroid carcinoma (PTC) at Shanghai Ninth People's Hospital from 2012 to 2016. All experiments to patients were approved by human ethics committee of Shanghai Ninth People's Hospital. The PTC cancer tissues and adjacent normal tissue were performed Immunohistochemical staining and analysis the relationship between LSD1 expression and Clinic pathologic of PTC.

Cell culture and siRNA transfection

Human normal thyroid follicular epithelial cell line Nthy-ori 3-1 and human papillary thyroid carcinoma cell lines B-CPAP and IHH-4 were cultured in RPMI-1640 culture media supplemented with 10% FBS (fetal bovine serum) and 1% PS (penicillin-streptomycin) (Sigma, USA) at 37°C with 5% CO₂. When cell were grown to almost 30% confluence, transfected either control siRNA or LSD1 siRNA in cells using lipofectamine 2000 reagent. After 48 h, collected cells.

Cell viability assay

CCK-8 assay was used to detected cell proliferation as previously described [25]. In short, cells were transfected with control siRNA or LSD1 siRNA or vector or FLAG-LSD1 for 48 h, follow by seeded 5×10³ cells/well in 96-well plates. Next cultivation for 24 h, add 10 μL of CCK-8 every 100 μl RPMI-1640 culture media and incubated 1 h in 37°C with 5% CO₂. Finally, absorbance at 450 nm represent cell number.

Clonogenic assay

Clonogenic assay was performed according previously published method [26]. Briefly, utilized LSD1 siRNA to knockdown LSD1 in B-CPAP and IHH-4 cells, and then plated 5×10³ cells into the 6-well plates in triplicate. Then, cultured the cells in RPMI-1640 containing 10% FBS for 12 days. After that, the cells were fixed with methanol and stained with 0.5% crystal violet. Finally, counted colonies under microscope (IX83, Olympus), and each colony was at least 40 cells.

Cell cycle

Transfection with siRNA and cultured cells in RPMI-1640 media containing 10% FBS for 48 h, subsequently blocking cell cycle in RPMI-1640 media without serum for 24 h, next released cell cycle for 24 h and collected cells. Fixed cells in 70% ethanol, and washed three times with PBS, finally stained with PI solution in the dark for 40 min. After that, analyzed DNA content and cell cycle by flow cytometry.

Quantitative RT-PCR (qRT-PCR)

Extracted total RNA from PTC cell lines and tissues by TRIzol Reagent (Invitrogen) according to manufacture protocol. Then, the purified RNA was used for synthesis cDNA (complementary DNA) by TransGen first-strand cDNA synthesis kit. The relative mRNA level of targets were detected by SYBR MIX in ABI PRISM 7500 system. GAPDH was used to as internal control. Primers sequence as follows: LSD1 sense primer, 5'-GAAGCATCTGAAGTAAAGCCA-3' and antisense primer, 5'-CAAAGTCATCCTGATCC-3'; TSHR forward: 5'-AGTGACTCATAGAAATTCGG-3' and reverse: 5'-TGTCCCATTGAAAGCATATCCT-3'; and GAPDH sense primer, 5'-CATTCCTGGTATGACAACGA-3' and antisense primer, 5'-TACATGGCAACTGTGAGGAG-3'.

Western blotting analysis

Cells were lysed with RIPA buffer containing protease inhibitor, cocktail (Sigma). centrifuged cells at 13,000 rpm for 15 min at 4°C for the protein collection. Collected the supernatants, and used BCA protein assay kit to determined protein concentration (Pierce). Subsequently, total 40 μg protein was subjected to a 10% SDS-PAGE and follow by transferred protein onto PVDF membrane (Millipore). Next blocked membranes in 5% non-fat milk at 4°C overnight or at room temperature for 1 h. Consequently, incubated with indicated antibodies at 4°C overnight. Next, washed membranes in TBST solution for three times, then incubated with secondary antibody (1:5000) for 1 h at room temperature. Finally, washed PVDF membranes and used ECL method to detected protein. Additionally, GAPDH (Sigma) as an internal control.

ChIP and qChIP assay using an EZ ChIP Kit (Millipore) to performed ChIP assay. In brief, sonicated cross-linked chromatin into 200-bp to 1000-bp fragments. The chromatin was

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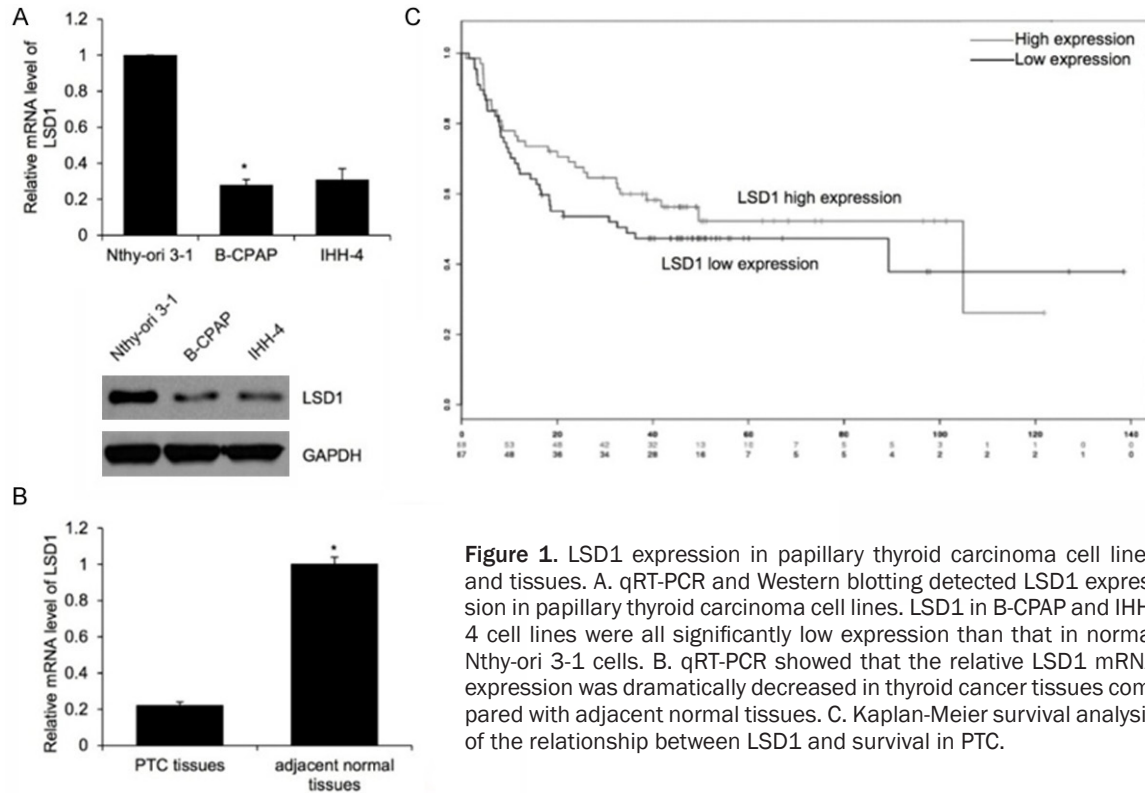


Figure 1. LSD1 expression in papillary thyroid carcinoma cell lines and tissues. A. qRT-PCR and Western blotting detected LSD1 expression in papillary thyroid carcinoma cell lines. LSD1 in B-CPAP and IHH-4 cell lines were all significantly low expression than that in normal Nthy-ori 3-1 cells. B. qRT-PCR showed that the relative LSD1 mRNA expression was dramatically decreased in thyroid cancer tissues compared with adjacent normal tissues. C. Kaplan-Meier survival analysis of the relationship between LSD1 and survival in PTC.

Table 1. Clinic pathologic variables in 146 PTC patients

Variables	No. (n=146)	LSD1 protein expression		P value
		Low (n=90)	High (n=56)	
Gender				
Male	86	50	36	0.297
Female	60	40	20	
Age				
≥ 50	71	47	24	0.271
< 50	75	43	32	
Tumor size				
Large (≥ 3 cm)	73	54	19	0.002
Small (≤ 3 cm)	73	36	37	
Pathological grade				
I-II	65	27	38	< 0.001
III-IV	81	63	18	
Lymph node metastasis				
Yes	79	66	13	< 0.001
No	67	24	43	
Drinking				
Yes	68	40	28	0.513
No	78	50	28	

immunoprecipitated using anti-H3K4me2 antibody. IgG was as negative control. Performed qRT-PCR by SYBR Green Mix (Roche).

Statistical analysis

In this study all experiments were at least conducted for three times. Used chi-square test to investigate the various clinic pathological characteristics of LSD1 expression. Comparisons between adjacent normal tissue and tumor tissue were used paired-samples *t* test. Data were analyzed using SPSS 19.0 and presented as the mean ± SD (standard deviation). *P*<0.05 as significant level.

Results

LSD1 expression in papillary thyroid carcinoma cell lines and tissues

In order to explore the function of LSD1 in papillary thyroid carcinoma, we first determined expression of LSD1 in papillary thyroid carcinoma cell lines and tissue. qRT-PCR was used to detected the

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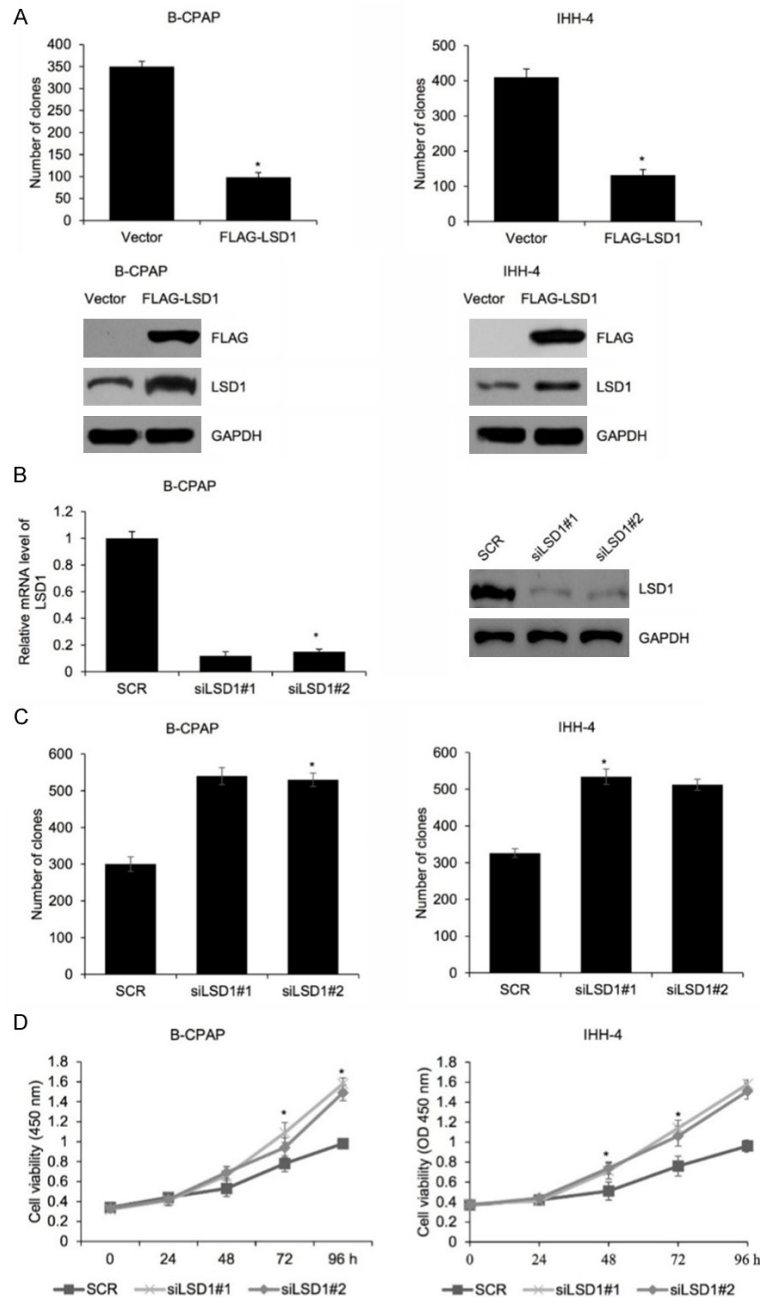


Figure 2. Knockdown LSD1 promoted cell proliferation. A. Over-expressed LSD1 in B-CPAP and IHH-4 cells, and performed clonogenic assay. Western blotting detected protein level of LSD1 in B-CPAP and IHH-4 cells. B. Utilized two different siRNA to knockdown LSD1 in B-CPAP cells and determine LSD1 by qRT-PCR and Western blotting. C. Clonogenic assay of LSD1 suppression on the cell proliferation. LSD1 depletion significantly increased the number in B-CPAP and IHH-4 cells. D. Performed CCK-8 assay to explore cell viability in B-CPAP and IHH-4 cells which LSD1 was silenced.

mRNA level of LSD1, results demonstrated that the relative LSD1 mRNA expression in papillary thyroid carcinoma cell lines B-CPAP and IHH-4 was dramatic down-regulation compared to that in human normal thyroid cell lines Nthy-ori

3-1, and protein level of LSD1 consistent with mRNA level of LSD1 (Figure 1A). Interestingly, relative LSD1 expressions in PTC tissues were also critically low than that in adjacent normal tissues (Figure 1B).

To further investigate the relationship between LSD1 and PTC, we analyzed LSD1 expression in 146PTC patients. IHC staining revealed that LSD1 protein was significant down-regulation in PTC compared to adjacent normal tissues. Interestingly, LSD1 expression negative correlated with tumor size ($P=0.002$), lymph node metastasis and Pathological grade ($P<0.001$). But, there were no significant correlation between LSD1 and other factors, such as drinking status, age and gender (Table 1).

Follow by, we explored the prognosis of LSD1 downregulation in PTC. Remarkably, survival curve showed that patients with low expression of LSD1 was significantly longer than that with high expression of LSD1 (Hazard Ratio =1.59, $P=0.019$, (Figure 1C).

Knockdown LSD1 promoted cell proliferation

Since LSD1 was down-regulation in PTC cell lines and LSD1 low expression negative relationship with tumor size, so we hypothesized that LSD1 regulate PTC growth. We first over expressed FLAG-LSD1 in IHH-4 cells and performed clonogenic assay to confirm the role of LSD1 on cell proliferation. The results revealed that the number of clones was significantly decreased in B-CPAP and IHH-4 cells which over-expressed LSD1 (Figure 2A). Follow by, we utilized two dif-

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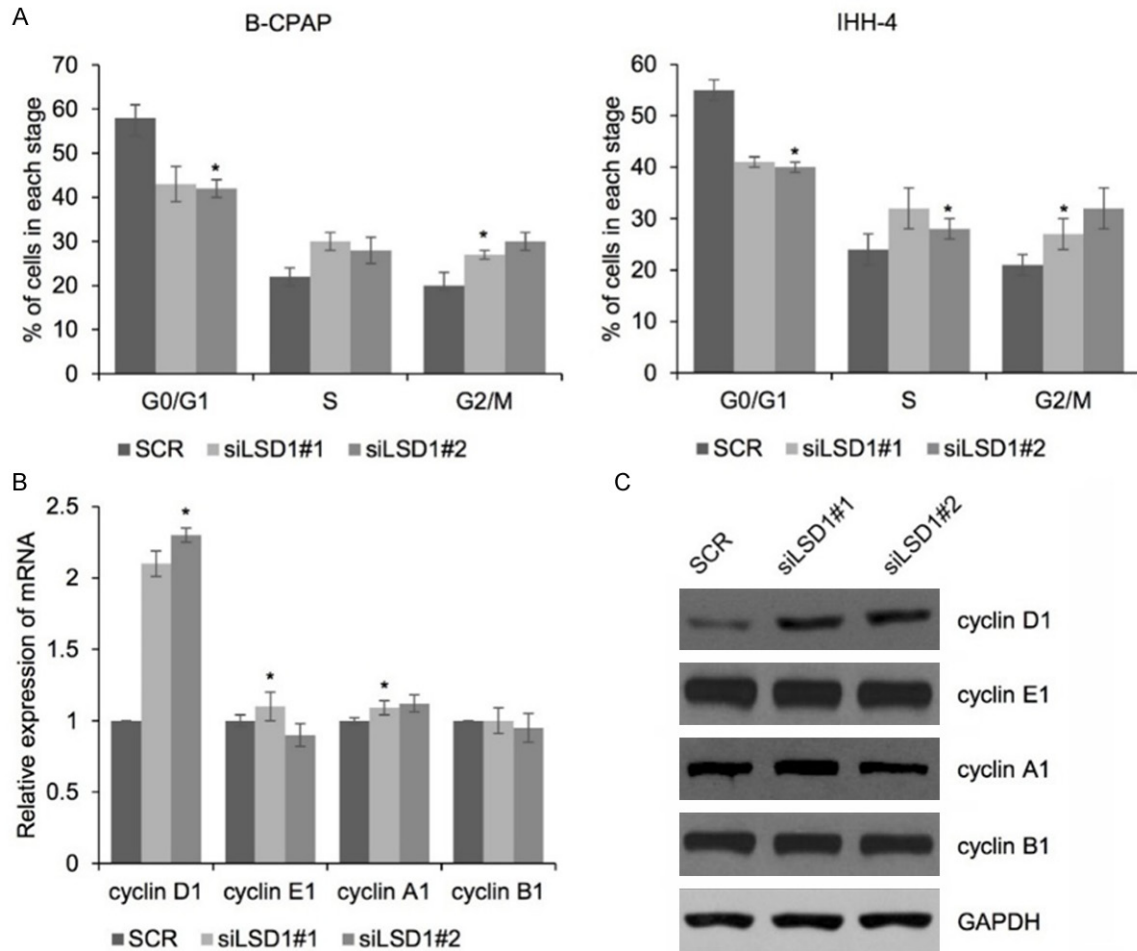


Figure 3. LSD1 expression modulated cell cycle. A. Flow cytometry revealed that LSD1 suppression significantly decreased the percentage of B-CPAP and IHH-4 cells in G0/G1 stage. B. qRT-PCR analysis showed that LSD1 suppression significantly increased mRNA level of cyclin D1 in B-CPAP cells, but performed not effects on cyclin A1, cyclin B1 and cyclin E1 expression. C. Western blotting analysis revealed that protein level of cyclin D1 was increased in B-CPAP cells which LSD1 was depleted. Whereas cyclin E1, cyclin A1 and cyclin B1 was no significantly changed.

ferent LSD1 siRNA to knockdown LSD1 in B-CPAP cell lines. After transfected for 2 days, collected cells and determine LSD1 siRNA efficiency. As shown in **Figure 2B**, LSD1 reduce almost 80% in cell which transfected LSD1 siRNA compared with control groups. And subsequently performed clonogenic assay, an opposed results can be seen in B-CPAP and IHH-4 cells which silenced LSD1. The number of clones was crucial increased in cells that LSD1 was depleted than control groups (**Figure 2C**). Moreover, CCK-8 assay was used to determine cell viability when LSD1 was depleted. We found that knockdown LSD1 make B-CPAP cells grew significantly faster than control group from 48 to 96 h (**Figure 2D**). Additionally, similar result can be seen in IHH-4 cells. In brief, LSD1 suppressed PTC cells proliferation.

LSD1 expression modulated cell cycle

To further investigate the effects of LSD1 on regulating papillary thyroid carcinoma cell proliferation, flow cytometry was performed to detected cell cycle profiling and cellular DNA content in B-CPAP and IHH-4 cells wh-ich LSD1 was depleted. Res-ults indicated that the number of cells at G1 stage was obviously decreased when LSD1 was knockdown in BCPAP and IHH-4 cell lines, compared with the control groups (**Figure 3A**), that mean when knock-down LSD1, cells were released from G1 stage. Accordingly, to verify whether LSD1 could indeed release the cell cycle in G0/G1 stage or not, mRNA expression and protein levels of cell cycle-related protein expression including cyclin A1, cyclin B1, cyclin D1 and cyclin E1

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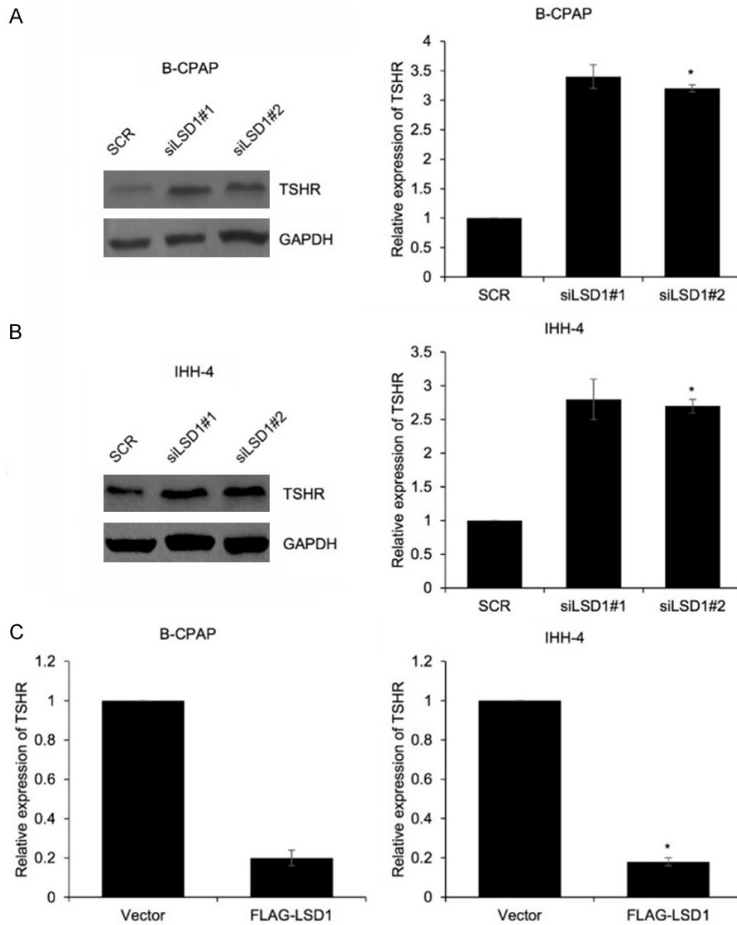


Figure 4. Effects of LSD1 suppression on TSHR expression. A. Transfected B-CPAP cells with LSD1 siRNA and detected protein and mRNA level of TSHR by Western blotting and qRT-PCR. TSHR was significantly increased compared with control group. B. Transfected IHH-4 cells with LSD1 siRNA and detected protein and mRNA level of TSHR by Western blotting and qRT-PCR. TSHR was significantly increased compared with control group. C. Over-expressed FLAG-LSD1 in B-CPAP and IHH-4 cells and detected mRNA level of TSHR by qRT-PCR. TSHR was dramatically decreased compared with control group.

were analyzed. Cyclin D1 was significantly increased when cells knockdown LSD1 compared to that in controls (**Figure 3B** and **3C**). When cyclin D1 expression increased, cells would faster enter S phase. Whereas, mRNA and protein level of cyclin A1, B1, and E1 were slightly changed in cells while knockdown LSD1 in B-CPAP cells (**Figure 3B** and **3C**).

Effects of LSD1 suppression on TSHR expression

There are several studies indicated that TSH and TSHR could regulated cell proliferation and function [20]. Therefore, we assumed that LSD1 influence PTC cell proliferation maybe through regulate TSHR. To verify our hypothe-

sis, we first silenced LSD1 in BCPAP and IHH-4 cells, and then analyzed the mRNA and protein level of TSHR in these kinds of thyroid cells by qRT-PCR and Western blotting. The results indicated that both mRNA level and protein levels of TSHR in B-CAP and IHH-4 cells were dramatically increased by silencing LSD1 compared to the controls (**Figure 4A** and **4B**). On the contrary, TSHR was significantly decreased in B-CAP and IHH-4 cells when over expressed LSD1 (**Figure 4C**). Together, above results suggested that LSD1 was negatively correlated with TSHR expression in PTC cells.

LSD1 suppresses TSHR transcription via demethylation H3K4me2

LSD1 is a histone demethylase catalyzing histone H3K4me to mediate gene silence. Histone methylation and demethylation are important epigenetic modification, and play critical function in cell process. So we hypothesized that LSD1 suppresses TSHR maybe via demethyltransferase H3K4me2. To verify this hypothesis, we performed ChIP assay with anti-H3K4me2 in B-CPAP cells which LSD1 was depleted. The result revealed that H3K4me2

at the TSHR promoter was obvious increased in B-CPAP cells which LSD1-silenced (**Figure 5A**). The similar results can be seen in IHH-4 cells (**Figure 5B**). It suggesting that H3K4me2 could targeting the promoter of TSHR to change TSHR expression. While reconstituted expression of RNAi-resistant LSD1 in B-CPAP cells, it was successful to reduce H3K4me2 at the LSD1. Together, all results showed that LSD1 decreases TSHR expression via modulating H3K4 demethylation.

Discussion

Recently, more and more evidence has suggested that histone modification play vital roles in multiple process of cancers development.

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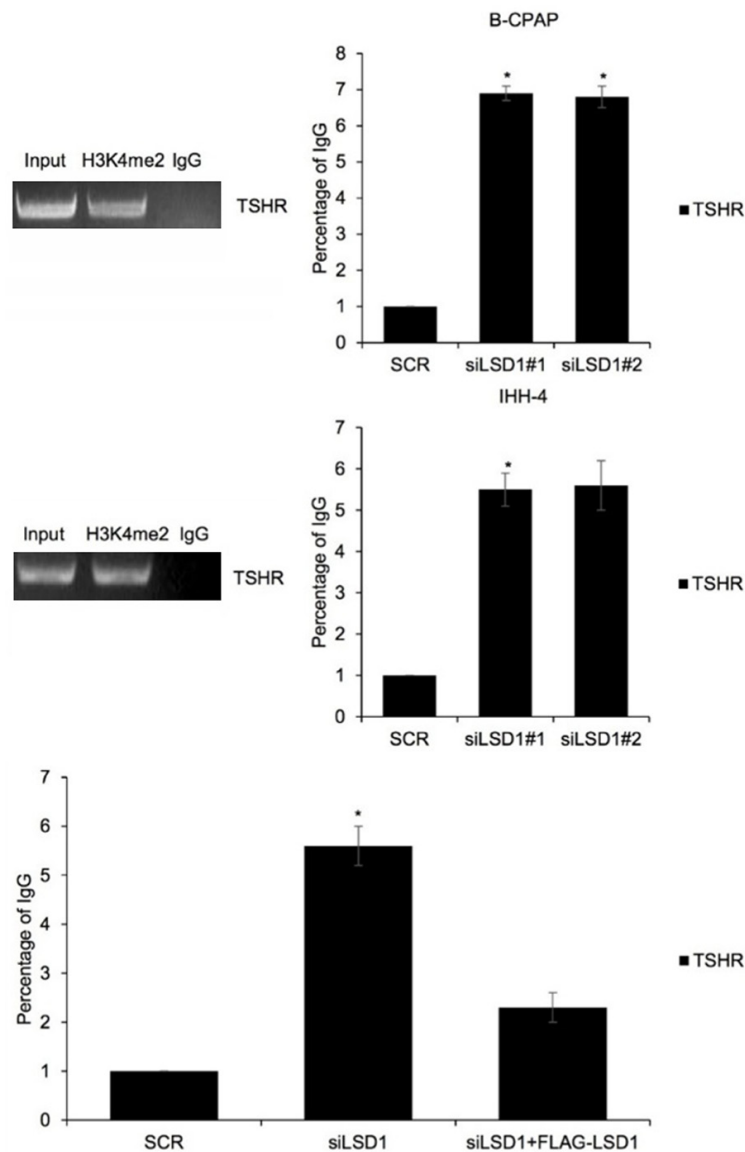


Figure 5. LSD1 suppresses TSHR transcription via demethylation H3K4me2. A. B-CPAP cells were transfected with LSD1 siRNA. Performed ChIP analyses with an H3K4me2 antibody. Follow by qChIP assay, result indicated that TSHR transcription was increased when LSD1 was silenced. B. IHH-4 cells were transfected with LSD1 siRNA. Performed ChIP analyses with an H3K4me2 antibody. Follow by qChIP assay, results indicated that TSHR transcription was increased when LSD1 was silenced. C. Reconstituted expression of RNAi-resistant LSD1 in B-CPAP/siLSD1 cell, next performed qChIP assay with an H3K4me2 antibody.

Many studies have shown that LSD1 influence tumor proliferation and invasion. However, the function of LSD1 in PTC have few known. In the present study, we determined the expression of LSD1 in PTC cell lines and tissues. The data demonstrated that LSD1 was dramatic down-regulation in papillary thyroid cancer tissues compared to adjacent normal tissues. The simi-

lar results can be seen in PTC cell lines IHH-4 and B-CPAP compared with human normal cell lines Nthy-ori 3-1. Therefore, we speculated that LSD1 depletion may be associated with papillary thyroid cancer pathogenesis. As our hypothesis, IHC assay showed that LSD1 expression is negative correlation with lymph node metastasis and tumor size. Moreover, LSD1 down-regulation also predict a poor prognosis.

Accordingly, we utilized LSD1 siRNA to knockdown LSD1 in B-CPAP and IHH-4 cells, and analyzed the effects of LSD1 expression on PTC cell proliferation. In agreement with previous studies, our results revealed that PTC cell proliferation was significantly promoted by LSD1 siRNA transfection, indicating that LSD1 could suppress PTC cell proliferation. Subsequently, we further analyzed the function of LSD1 on PTC cell cycle. Our data demonstrated that cell cycle was released from G0/G1 stage in IHH-4 and B-CPAP cell which knockdown LSD1. Cell cycle is promoted by cyclin D1 accumulate in papillary thyroid cancer [27, 28]. So we detected cell cycle associated protein expression. We found cyclin D1 expression was also significantly increased by knockdown LSD1. Above results suggesting that cyclin D1 up-regulation was correlated to PTC cells proliferation promotion via targeting the cell cycle at G0/G1 stage.

TSH accord to bind TSHR to altered thyroid cell proliferation, and aberrant expression of TSHR would result in Grave's disease or thyroid cancer pathogenesis [19, 29, 30]. We found both mRNA and protein levels of TSHR were significant up-regulation when LSD1 siRNA transfection in IHH-4 and B-CPAP cells, indicating that silencing LSD1 may promotes the development

of PTC by up-regulating TSHR expression. An opposite result can be seen, over-express LSD1 would suppress PTC cell proliferation through down-regulated TSHR.

DNA methylation and histone modifications have been revealed that influences tumorigenesis in thyroid cancer via epigenetic alteration [22]. ChIP assay was performed to investigate whether LSD1 regulated TSHR transcription, and our data indicated that TSHR was significantly increased by silencing LSD1 in IHH-4 and B-CPAP cell lines, suggesting that H3K4me2 binding to TSHR promoter. Taken together, we speculated that LSD1 suppresses TSHR expression through demethylation H3K4.

In conclusion, we found LSD1 down-regulation in PTC and modulated cancer cell proliferation. Over-expressed LSD1 result in TSHR transcriptional suppression via demethylation H3K4me2, thereby inhibit the cell proliferation. Our study provide a new therapeutic target for thyroid cancer.

Disclosure of conflict of interest

None.

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