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Immunohistochemical expression of RBP2 and LSD1 in papillary thyroid carcinoma

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Abstract

Purpose: To investigate the prognostic significance of LSD1 and RBP2 expression in patients with papillary thyroid carcinoma. *Materials and Methods*: LSD1 and RBP2 expressions were detected by immunohistochemistry in surgically resected samples from thyroid adenoma, papillary thyroid carcinoma and paracancerous tissues. *Results*: To be members of histone demethylases, LSD1 and RBP2 were both localized mainly to the thyroid cell nucleus. Despite the fact that both RBP2 and LSD1 expressions were higher in papillary thyroid carcinoma than in paracancerous tissues (U=-3.855, p=0.000; U=-5.575, p=0.000) and thyroid adenoma (U=-1.972, p=0.049; U=-3.190, p=0.001), they did not show us statistical correlation (r=-0.149, p=0.270). Like LSD1 (U=-2.286, p=0.022), RBP2 expression was less frequently in paracancerous tissues than in thyroid adenoma (U=-1.985, p=0.047). Neither LSD1 nor RBP2 expression was significantly associated with age, gender, stage status, tumor size, and lymph node metastases (p>0.05). *Conclusions*: Both LSD1 and RBP2 are well related with the occurrence and malignant transformation of papillary thyroid carcinoma. Though the positive expression of both LSD1 and RBP2 can be used to estimate the potentiality of thyroid carcinoma and help for the adjuvant treatment, LSD1 is a more sensitive molecular marker than RBP2 on thyroid cancer diagnosis.

Keywords: papillary thyroid carcinoma, lysine-specific demethylase 1, retinoblastoma binding protein.

Introduction

Thyroid cancer is the most common malignancy of the endocrine system [1]. Thyroid cancer can be histologically classified into papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), anaplastic thyroid cancer (ATC), and medullary thyroid cancer (MTC), which account for approximately 80%, 15%, 2%, and 3% of all thyroid malignancies, respectively [2].

During the past several decades, an increasing incidence rate of thyroid cancer had been observed in several countries, including USA [3], Romania [4], Denmark [5], Great Britain [6], New Zealand [7] and China [8]. Although many researches have been carried out on oncogenic genetic alterations, the molecular mechanism of thyroid cancer remains controversial. Recent genetic analysis focus on RET, Ras, BRAF, PIK3CA, and PTEN. However, published studies remain indeterminate.

The histone modifying enzymes play a key role in regulating gene transcription by mediating chromatin reconfiguration. LSD1 (Lysine Specific Demethylase 1) is the first demethylase to be discovered specifically to convert dimethylated H3K4 or H3K9 [9]. RBP2 (Retinoblastoma Binding Protein) is a newly identified histone demethylase targeting H3K4 me3/me2 for demethylation [10].

Although LSD1 and RBP2 are essential for the development of cell, the pathological roles of their disturbance in thyroid tumor remain unclear completely. Here, we try to demonstrate important roles of LSD1 and RBP2 in thyroid tumor.

Materials and Methods

Tissue specimens studied

Ninety-five cases of thyroid tissue were enrolled in this study, including 15 cases of thyroid adenoma, 57 cases of papillary thyroid carcinoma (PTC), and 23 cases of paracancerous tissues. The patients with PTC used in this study had an average age of 46.7±14.5 years (16 males and 41 females). Fifteen adenomas were from patients with an average age of 45.1±7.7 years (three males and 12 females).

Tissue specimens were obtained *via* surgery between 2009.01.03–2010.12.22 at Affiliated Hospital of Jining Medical University in Jining, China. Each case was evaluated for clinical parameters including age, gender, stage status, tumor size, and lymph node metastases. The pathological diagnoses of the samples were independently established according to the *World Health Organization* classification by two experienced pathologists. The formalin-fixed paraffin-embedded tissue sections were used following the approval of the Ethics Committee of Affiliated Hospital of Jining Medical University, after informed consent was obtained from the patients.

Immunohistochemistry staining

Archival paraffin-embedded tumor tissues were analyzed for LSD1 and RBP2 expressions. The sections were deparaffinized and rehydrated according to routine protocol. Antigen retrieval was done in 10 mM citrate buffer inside a microwave pressure cooker for 10 minutes. Then, the sections were treated with 0.3% hydrogen

peroxide to quench endogenous peroxidase activity for 30 minutes at 37^0C. Then, these slides were incubated for 30 minutes with goat anti-rabbit immunoglobulin after being washed with Tris-buffered sodium chloride solution. All samples were incubated for 24 hours at 4^0C with the specific primary monoclonal antibodies. Primary antibodies were used as follows: LSD1: 1:400 dilution (Cell Signaling Technology, Danvers, MA, USA; #2184S); RBP2: 1:100 dilution (Cell Signaling Technology, USA; #3876S). Following a TBS wash, the slides were incubated with a polyperoxidase-conjugated anti-rabbit secondary antibody (K116820C, Peking, China) for 30 minutes. After washing in TBS, the sections would be visualized with DAB chromogen and counterstained with Hematoxylin. Primary antibodies were replaced by TBS in the negative control.

Evaluation of score

Quantitative evaluation was performed by counting of the percentage of positively stained cells, using an eyepiece graticule facilitate cell counting, first under low magnification (×100), then at a higher magnification (×400), at which a minimum of 1000 cells were counted in the area with positive staining. The extent of positivity was scored as follows: 0, no staining; 1, pale yellow; 2, claybank; 3, brown. The percentage of positivity was scored as follows: 0, <5%; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, >75%. The final score was obtained by multiplying the extent of positivity and intensity scores, producing a range from 0 to 12. The intensity was scored as follows: 0–3, negative; 4–6, weak; 7–9, moderate; and 10–12, strong.

Statistical analyses

Statistical comparisons of data were performed by nonparametric Mann–Whitney *U*-test, Wilcoxon signed-rank test, and Spearman's correlation analysis on SPSS 13.0 software. The level of statistical significance was set at $p<0.05$.

Results

Immunohistochemical analysis demonstrated that less LSD1 (Figure 1) expressed in paracancerous tissues than in thyroid adenoma (U=-2.286, p=0.022), and more LSD1 expressed in PTC (94.7%) than in paracancerous tissues (34.8%) (U=-5.575, p=0.000) and thyroid adenoma (73.3%) (U=-3.190, p=0.001). There was no difference between cases with negative lymph node metastases and with positive lymph nodes (U=-0.364, p=0.716). The expression of LSD1 was not significantly associated with clinical stage status, age, gender, and tumor size ($p>0.05$, Table 1).

Table 1 – ***Expression of RBP2 and LSD1 according to clinical pathological parameters***

Characteristics	*n*	Expression of RBP2				*U*/*p*	Expression of LSD1				*U*/*p*
		-	+	++	+++		-	+	++	+++	
Pathologic type											
▪ *paracancerous*	23	22	1	0	0	16.983/ 0.000	15	5	2	1	35.332/ 0.000
▪ *thyroid adenoma*	15	11	3	1	0		4	6	3	2	
▪ *thyroid carcinoma*	57	28	11	9	9		3	9	21	24	
Clinical stage											
▪ *I*	16	10	3	3	0	3.751/ 0.290	2	2	7	5	1.359/ 0.715
▪ *II*	18	9	4	2	3		0	4	6	8	
▪ *III*	5	2	2	0	1		0	1	1	3	
▪ *IV*	18	7	2	4	5		1	2	7	8	
Age [years]											
▪ *<45*	28	11	7	6	4	-0.000/ 1.000	1	3	7	4	-1.154/ 0.248
▪ *≥45*	29	17	4	3	5		2	6	14	20	
Lymph node metastasis											
▪ *positive*	17	10	2	3	2	-0.787/ 0.431	1	1	8	7	-0.364/ 0.716
▪ *negative*	40	18	9	6	7		2	8	13	17	
Sex											
▪ *male*	16	8	4	2	2	-0.343/ 0.731	1	5	5	5	-1.512/ 0.130
▪ *female*	41	20	7	7	7		2	4	16	19	
Tumor size											
▪ *T1*	22	11	6	4	1	3.753/ 0.289	1	4	9	8	1.020/ 0.796
▪ *T2*	21	11	4	2	4		1	4	5	11	
▪ *T3*	8	5	0	1	2		1	0	5	2	
▪ *T4*	6	1	1	2	2		0	1	2	3	

Immunohistochemical studies revealed that RBP2 expression (Figure 2) was higher (50.9%) in papillary thyroid carcinoma than in paracancerous tissues (4.3%) (U=-3.855, p=0.000) and thyroid adenoma (26.7%) (U=-1.972, p=0.049). In addition, RBP2 expression was less frequently in paracancerous tissues than in thyroid adenoma (U=-1.985, p=0.047). The expression of RBP2 was not significantly associated with clinical stage status, age, gender, tumor size, and lymph nodes metastasis ($p>0.05$).

Though both LSD1 and RBP2 were localized mainly to nucleus of the thyroid cell, they did not show us a statistical correlation in PTC (r=-0.149, p=0.270, Table 2), in thyroid adenoma (r=0.300, p=0.278, Table 3) or in paracancerous tissues (r=-0.152, p=0.488, Table 4). Among PTC cases, most of them are LSD1 positive with a rate of 94.7%, of which about 44.4% were +++ score. Nearly half of them are RBP2 positive (50.9%) among PTC cases, of which about 31.0% were +++ score. Wilcoxon signed-rank test showed us that expression of LSD1 was higher than that of RBP2 in PTC (U=-4.717, p=0.000, Table 2). Wilcoxon signed-rank test showed us that RBP2 expression was less than LSD1 expression in thyroid adenoma (U=-2.667, p=0.008, Table 3).

Table 2 – ***Correlation between LSD1 and RBP2 in PTC***

Characteristic		Expression of LSD1					r/p	U/p
		-	+	++	+++	n		
Expression of RBP2	-	3	2	8	15	28	-0.149/ 0.270	-4.717/ 0.000
	+	0	3	4	4	11		
	++	0	2	6	1	9		
	+++	0	2	3	4	9		
	n	3	9	21	24	57		

Table 3 – ***Correlation between LSD1 and RBP2 in thyroid adenoma***

Characteristic		Expression of LSD1					r/p	U/p
		-	+	++	+++	n		
Expression of RBP2	-	3	5	3	0	11	0.300/ 0.278	-2.667/ 0.008
	+	1	1	0	1	3		
	++	0	0	0	1	1		
	+++	0	0	0	0	0		
	n	4	6	3	2	15		

Table 4 – ***Correlation between LSD1 and RBP2 in paracancerous tissues***

Characteristic		Expression of LSD1					r/p	U/p
		-	+	++	+++	n		
Expression of RBP2	-	14	5	2	1	22	-0.152/ 0.488	-2.326/ 0.020
	+	1	0	0	0	1		
	++	0	0	0	0	0		
	+++	0	0	0	0	0		
	n	15	5	2	1	23		

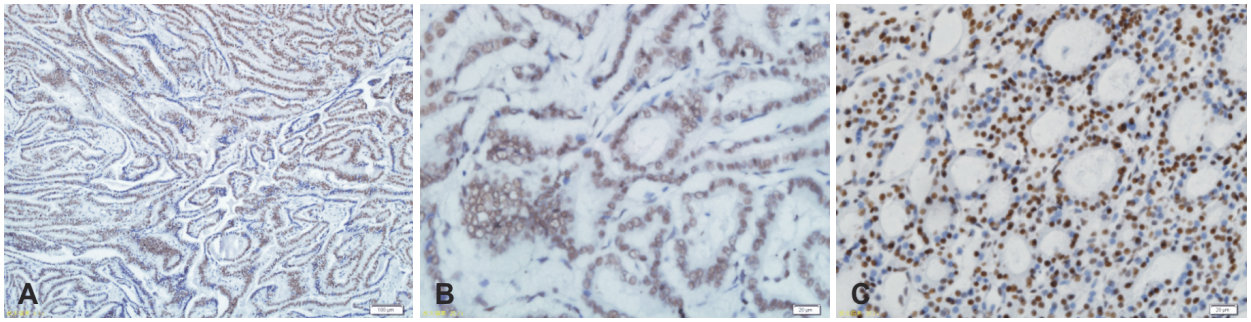

Figure 1 – ***(A–C) LSD1 expression and distribution in papillary thyroid carcinoma tissue and thyroid adenoma, detected by IHC staining. LSD1 positive stains were located in the cell nuclei. (A and B) LSD1 expression was up-regulated in PTC. (C) LSD1 expression was up-regulated in thyroid adenoma. A, ×100; B, ×400; C, ×200.***

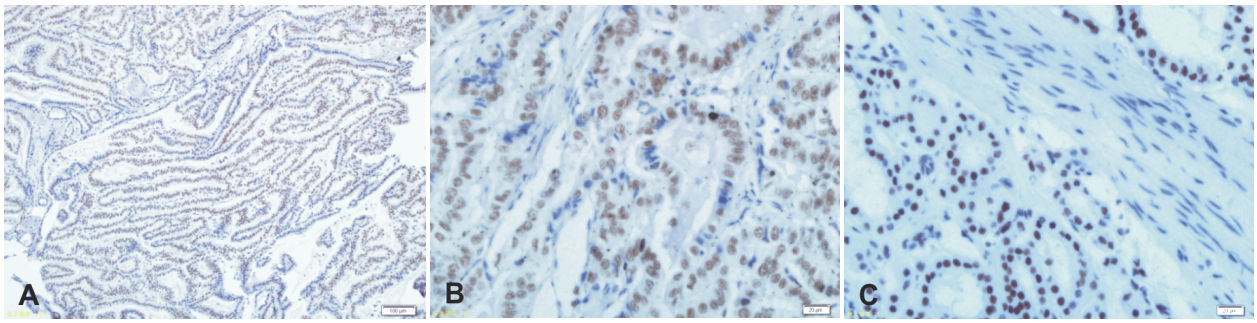

Figure 2 – ***(A–C) RBP2 expression and distribution in papillary thyroid carcinoma tissue and thyroid adenoma, detected by IHC staining. RBP2 positive stains were located in the cell nuclei. (A and B) RBP2 expression was up-regulated in PTC. (C) RBP2 expression was up-regulated in thyroid adenoma. A, ×100; B, ×400; C, ×400.***

Discussion

Of the whole endocrine system, thyroid carcinoma is the most common malignancy and is increasing in incidence in the world. Papillary thyroid cancer is the most common of all thyroid carcinomas. Clinical markers are needed for prognoses for PTC.

In recent years, epigenetic has become a hot topic in cancer research. The balance of methylation and demethylation in epigenetic modification affects gene expression and cellular activity. Studies have demonstrated that aberrant histone lysine methylation in cancer is associated not only with the repression of chromatin related to specific genes, but also with the repression of large chromosomal regions. Epigenetic changes in LSD1 have been shown to play a key role in carcinogenesis. New research demonstrated that LSD1 inhibited the invasive growth of breast cancer cells *in vitro* and was downregulated in breast carcinoma [11]. Aberrant expression of LSD1 has been shown in many types of cancers [12, 13], including lung cancer [14], neuroblastoma [15], breast cancer [16], and Ewing sarcoma [17]. LSD1 expression was upregulated strongly in non-small cell lung cancer patients. Interruption of LSD1 using siRNA or Pargyline, suppressed proliferation, invasion and metastasis of lung cancer cells lines [14]. LSD1 was involved in keeping the malignant phenotype of neuroblastoma cells; knockdown of LSD1 decreased cellular growth, reduced neuroblastoma xenograft growth *in vivo* [15]. Researchers found that overexpression of LSD1 was associated with poor prognosis in ER-negative breast cancers. Small interfering RNA-mediated knockdown of LSD1 showed that LSD1 was recruited to the promoters of some genes like p21, ERBB2, and CCNA2; LSD1 could be regarded as a predictive biomarker for diagnosis and tumor therapy [16].

Our research demonstrated that less LSD1 expressed in paracancerous tissues than in thyroid adenoma and papillary thyroid carcinoma, which means LSD1 plays a very important role in occurrence and progression of thyroid tumor. Further studies indicated LSD1 plays a very important role in malignant transformation in PTC: Compared with expression in thyroid adenoma, more LSD1 expressed in PTC, and more strong positivity showed in PTC.

RBP2, also called KDM5A, was considered as a regulation of transcription and differentiation by the retinoblastoma tumor suppressor protein [18]. All members of KDM5 cluster catalyze the demethylation of the same histone mark, though they seem to have different expression profiles and presence in distinct protein complexes [19, 20]. The function of RBP2 in human cancers is poorly understood, and the few results are contradictory. Recently, studies indicated that RBP2 was upregulated in gastric cancer [21]. RBP2 triggers cellular senescence of gastric cancer cells through binding to the promoters of cyclin-dependent kinase inhibitors p16, p21, and p27 and removing H3K4/me3 at these sites [21]. Analysis of breast carcinoma shows that downregulation of RBP2 enhanced progesterone receptor expression, while overexpression of RBP2 suppressed progesterone receptor promoter activity [22]. Recent analyses showed that RBP2 contribute to tumorigenesis. Genetic ablation of RBP2 decreases tumor formation and prolongs survival in Rb1 (+/-) mice and Men1-defective mice [23]. Knockdown of RBP2 by small interfering RNA significantly elevated mRNA expression of osteogenesis-associated genes, promoted osteogenic differentiation of human adipose-derived stromal cells *in vitro* and *in vivo* [24]. Present study showed that RBP2 binds to the hTERT promoter, demethylates H3K4, and regulates expression of the hTERT gene in normal or differentiated malignant cells [25].

Like LSD1, RBP2 expression was less frequently in paracancerous tissues than in thyroid adenoma and papillary thyroid carcinoma, which means RBP2 also plays a very important role in occurrence and progression of thyroid tumor. Neither LSD1 nor RBP2 expression was significantly associated with age, gender, stage status, tumor size, and lymph node metastases.

Though both LSD1 and RBP2 were localized mainly to nucleus of the thyroid cell, they did not show us a statistical correlation in PTC or in thyroid adenoma. The positive expression of both LSD1 and RBP2 can be used to estimate the potentiality of thyroid carcinoma and help for the adjuvant treatment. The expression of LSD1 was higher than that of RBP2 in PTC and thyroid adenoma, which means LSD1 is a more sensitive molecular marker than RBP2 on thyroid cancer diagnosis.

Histone methylation is considered as an important type of histone modification defining differentiation of cells. A better understanding of this epigenetic mechanism would provide new insights into cancer therapy.

Conclusions

Our study demonstrated that both RBP2 and LSD1 played a very important role in occurrence, progression, and malignant transformation in PTC. Neither LSD1 nor RBP2 expression was significantly associated with age, gender, stage status, tumor size, and lymph node metastases. No statistical correlation was found between LSD1 and RBP2 on thyroid tissues. Though the positive expression of both LSD1 and RBP2 can be used to estimate the potentiality of thyroid carcinoma and help for the adjuvant treatment, LSD1 is a more sensitive molecular marker than RBP2 on thyroid cancer diagnosis.

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References

[1] Sherman SI, *Thyroid carcinoma*, Lancet, 2003, 361(9356): 501–511.

[2] Hundahl SA, Fleming ID, Fremgen AM, Menck HR, *A National Cancer Data Base report on 53,856 cases of thyroid carcinoma treated in the U.S., 1985–1995 [see comments]*, Cancer, 1998, 83(12):2638–2648.

[3] Li N, Du XL, Reitzel LR, Xu L, Sturgis EM, *Impact of enhanced detection on the increase in thyroid cancer incidence in the United States: review of incidence trends by socioeconomic status within the surveillance, epidemiology, and end results registry, 1980–2008*, Thyroid, 2013, 23(1):103–110.

[4] Cătană R, Boilă A, Borda A, *Thyroid cancer profile in Mures County (Romania): a 20 years study*, Rom J Morphol Embryol, 2012, 53(4):1007–1012.
[5] Londero SC, Krogdahl A, Bastholt L, Overgaard J, Pedersen HB, Frisch T, Bentzen J, Pedersen PU, Christiansen P, Godballe C, *Papillary thyroid carcinoma in Denmark 1996–2008: an investigation of changes in incidence*, Cancer Epidemiol, 2013, 37(1):e1–e6.
[6] McNally RJ, Blakey K, James PW, Gomez Pozo B, Basta NO, Hale J, *Increasing incidence of thyroid cancer in Great Britain, 1976–2005: age-period-cohort analysis*, Eur J Epidemiol, 2012, 27(8):615–622.
[7] Albert BB, Cutfield WS, Webster D, Carll J, Derraik JG, Jefferies C, Gunn AJ, Hofman PL, *Etiology of increasing incidence of congenital hypothyroidism in New Zealand from 1993–2010*, J Clin Endocrinol Metab, 2012, 97(9):3155–3160.
[8] Xiang J, Wu Y, Li DS, Shen Q, Wang ZY, Sun TQ, An Y, Guan Q, *New clinical features of thyroid cancer in eastern China*, J Visc Surg, 2010, 147(1):e53–e56.
[9] Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA, Casero RA, Shi Y, *Histone demethylation mediated by the nuclear amine oxidase homolog LSD1*, Cell, 2004, 119(7): 941–953.
[10] Christensen J, Agger K, Cloos PA, Pasini D, Rose S, Sennels L, Rappsilber J, Hansen KH, Salcini AE, Helin K, *RBP2 belongs to a family of demethylases, specific for tri- and dimethylated lysine 4 on histone 3*, Cell, 2007, 128(6): 1063–1076.
[11] Wang Y, Zhang H, Chen Y, Sun Y, Yang F, Yu W, Liang J, Sun L, Yang X, Shi L, Li R, Li Y, Zhang Y, Li Q, Yi X, Shang Y, *LSD1 is a subunit of the NuRD complex and targets the metastasis programs in breast cancer*, Cell, 2009, 138(4): 660–672.
[12] Rotili D, Mai A, *Targeting histone demethylases: a new avenue for the fight against cancer*, Genes Cancer, 2011, 2(6):663–679.
[13] Hayami S, Kelly JD, Cho HS, Yoshimatsu M, Unoki M, Tsunoda T, Field HI, Neal DE, Yamaue H, Ponder BA, Nakamura Y, Hamamoto R, *Overexpression of LSD1 contributes to human carcinogenesis through chromatin regulation in various cancers*, Int J Cancer, 2011, 128(3): 574–586.
[14] Lv T, Yuan D, Miao X, Lv Y, Zhan P, Shen X, Song Y, *Over-expression of LSD1 promotes proliferation, migration and invasion in non-small cell lung cancer*, PLoS One, 2012, 7(4):e35065.
[15] Schulte JH, Lim S, Schramm A, Friedrichs N, Koster J, Versteeg R, Ora I, Pajtler K, Klein-Hitpass L, Kuhfittig-Kulle S, Metzger E, Schüle R, Eggert A, Buettner R, Kirfel J, *Lysine-specific demethylase 1 is strongly expressed in poorly differentiated neuroblastoma: implications for therapy*, Cancer Res, 2009, 69(5):2065–2071.
[16] Lim S, Janzer A, Becker A, Zimmer A, Schüle R, Buettner R, Kirfel J, *Lysine-specific demethylase 1 (LSD1) is highly expressed in ER-negative breast cancers and a biomarker predicting aggressive biology*, Carcinogenesis, 2010, 31(3): 512–520.
[17] Sankar S, Bell R, Stephens B, Zhuo R, Sharma S, Bearss DJ, Lessnick SL, *Mechanism and relevance of EWS/FLI-mediated transcriptional repression in Ewing sarcoma*, Oncogene, 2012 Nov 26.
[18] Klose RJ, Yan Q, Tothova Z, Yamane K, Erdjument-Bromage H, Tempst P, Gilliland DG, Zhang Y, Kaelin WG Jr, *The retinoblastoma binding protein RBP2 is an H3K4 demethylase*, Cell, 2007, 128(5):889–900.
[19] Blair LP, Cao J, Zou MR, Sayegh J, Yan Q, *Epigenetic regulation by lysine demethylase 5 (KDM5) enzymes in cancer*, Cancers (Basel), 2011, 3(1):1383–1404.
[20] Sharma SV, Lee DY, Li B, Quinlan MP, Takahashi F, Maheswaran S, McDermott U, Azizian N, Zou L, Fischbach MA, Wong KK, Brandstetter K, Wittner B, Ramaswamy S, Classon M, Settleman J, *A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations*, Cell, 2010, 141(1):69–80.
[21] Zeng J, Ge Z, Wang L, Li Q, Wang N, Björkholm M, Jia J, Xu D, *The histone demethylase RBP2 is overexpressed in gastric cancer and its inhibition triggers senescence of cancer cells*, Gastroenterology, 2010, 138(3):981–992.
[22] Stratmann A, Haendler B, *The histone demethylase JARID1A regulates progesterone receptor expression*, FEBS J, 2011, 278(9):1458–1469.
[23] Lin W, Cao J, Liu J, Beshiri ML, Fujiwara Y, Francis J, Cherniack AD, Geisen C, Blair LP, Zou MR, Shen X, Kawamori D, Liu Z, Grisanzio C, Watanabe H, Minamishima YA, Zhang Q, Kulkarni RN, Signoretti S, Rodig SJ, Bronson RT, Orkin SH, Tuck DP, Benevolenskaya EV, Meyerson M, Kaelin WG Jr, Yan Q, *Loss of the retinoblastoma binding protein 2 (RBP2) histone demethylase suppresses tumorigenesis in mice lacking Rb1 or Men1*, Proc Natl Acad Sci U S A, 2011, 108(33):13379–13386.
[24] Ge W, Shi L, Zhou Y, Liu Y, Ma GE, Jiang Y, Xu Y, Zhang X, Feng H, *Inhibition of osteogenic differentiation of human adipose-derived stromal cells by retinoblastoma binding protein 2 repression of RUNX2-activated transcription*, Stem Cells, 2011, 29(7):1112–1125.
[25] Ge Z, Li W, Wang N, Liu C, Zhu Q, Björkholm M, Gruber A, Xu D, *Chromatin remodeling: recruitment of histone demethylase RBP2 by Mad1 for transcriptional repression of a Myc target gene, telomerase reverse transcriptase*, FASEB J, 2010, 24(2):579–586.

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