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SETDB2 Inhibits Tumor Growth and Promotes Differentiated Markers Expression in Thyroid Cancer

Qi Jiang[1], Qingzhu Duan[2,3], Haifeng Luo[2], Feiliyan Maimaiti[2,3], Tianci Shen[2], Zhikun Lin[2], Jian Jiang[4], Jiangning Gu[2*], Dan Chen[1*]

[1]Department of Pathology, the First affiliated Hospital of Dalian Medical University, Dalian, Liaoning Province, China
[2]Department of Hepatobiliary Surgery, the First affiliated Hospital of Dalian Medical University, Dalian, Liaoning Province, China
[3]Department of Neurosurgery, the First affiliated Hospital of Dalian Medical University, Dalian, Liaoning Province, China
[4]Deparment of Emergency, the First affiliated Hospital of Dalian Medical University, Dalian, Liaoning Province, China

[*]**Corresponding author:** Dan chen, Department of Pathology, the First affiliated Hospital of Dalian Medical University, Dalian, Liaoning Province, China. Dalian 116011, Liaoning Province, People's Republic of China, Email: chendan78@dmu.edu.cn
Jiangning Gu, Department of Hepatobiliary Surgery, the First affiliated Hospital of Dalian Medical University, Dalian, Liaoning Province, China. Dalian 116011, Liaoning Province, People's Republic of China, Email: gujiangning@dmu.edu.cn

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Abstract

Thyroid cancer (TC) is one of the most prevalent endocrine malignancies with an increasing incidence worldwide. Though therapeutic effect is relatively good in the vast majority of TC patients, the anaplastic thyroid cancer (ATC) remains one of the most malignant tumors. This study aimed to investigate the role of SETDB2, an epigenetic regulator, in the dedifferentiation of TC cells. The expression of SETDB2 was detected in different histological types of TC and adjacent non-tumor tissues at mRNA and protein level. Simultaneously, the association between SETDB2 and thyroid differentiation markers (Thyroglobulin, TTF1 and PAX8) was analyzed to evaluate its role in TC differentiation. Furthermore, papillary thyroid cancer (PTC) cell lines BCPAP and ATC cell lines BHT101 were used to explore the function of SETDB2 in vitro. Our results indicated that the expression of SETDB2 was significantly reduced in TC compared to adjacent non-tumor tissues, while in ATC, it was much lower than that in PTC. Moreover, SETDB2 was highly associated with multiplicity and TNM stage. In vitro experiments revealed that overexpression of SEDTB2 could inhibit tumor growth while promote the expression of thyroid differentiation markers, and immunohistochemistry results also validated that the expression of SETDB2 was positively correlated with thyroid differentiation markers in clinical samples. Collectively, our data demonstrated that SETDB2 was differentially expressed in TC, and overexpression of SETDB2 suppressed tumor growth and promoted thyroid differentiation markers expression, which indicated that SETDB2 may be involved in the differentiation of TC and may serve as a potential therapeutic marker.

Keywords : SETDB2, Thyroid, Cancer, Dedifferentiation, Epigenetic Modification, Histone Methylation

Introduction

Thyroid cancer (TC) is one of the most common malignancies of the endocrine system, with an increasing incidence in recent years worldwide [1]. According to the latest statistics, the number of new cases of TC is approximately 586,202 in 2020, ranking 9th among all malignancies, even 5th among females [2]. TC mainly originates from two types of cells, the vast majority (>95%) from follicular epithelial cells and the others from parafollicular C cells [3]. Follicular cells-derived TC includes papillary thyroid cancer (PTC, 85%), follicular thyroid cancer (FTC, 2-5%), poorly differentiated thyroid cancer (PDTC, 6%) and anaplastic thyroid cancer (ATC, 1-2%) [4]. PTC usually has a relatively better prognosis, however, local recurrences and long-distance metastases are main critical contributors associated with the prognosis of PTC patients [5]. While ATC is the most aggressive histopathological type of TC, whose overall survival is less than 6 months [6]. Previous studies have proposed that the tumorigenesis of ATC may be a multistep process with a biological transformation from PTC to ATC [7; 8]. To date, the pathogenesis of ATC remains elusive and there is not yet an effective treatment strategy [9]. Thus, it is urgent to explore the tumorigenesis mechanism of ATC.

Epigenetic modification is extremely prevalent, and accumulating evidence demonstrates that epigenetic dysregulation plays an important role in the occurrence and development of TC [10]. Histone methylation is one of the most important approaches for epigenetic modulation of gene expression, and its mechanism is complex [11]. For example, trimethylation of histone H3 lysine 4 (H3K4) and H3K36 is involved in gene transcriptional activation, whereas trimethylation of H3K9 and H3K27 are associated with transcriptional suppression [12]. The potential contributions of histone methylation toward thyroid carcinogenesis are gaining attention [13]. Several recent studies suggest an association between histone methylation and dedifferentiation of TC [14-16].

SETDB2 (SET domain, bifurcated 2), a member of the SUV39 sub-family of the H3K9 methyltransferase family, has conserved SET structural domain that specifically catalyzes histone H3K9 trimethylation [17]. SETDB2 exerts its functions in several biological processes, including proliferation, inflammation and cell differentiation [18]. SETDB2 has been identified to be upregulated in multiple solid tumors, including gastric cancer [19], breast cancer [20], renal cell tumor [21] and hematologic tumors, e.g. acute lymphoblastic leukemia [22]. Moreover, overexpression of SETDB2 predicted poor prognosis in various malignancies [19; 21; 22]. However, the expression level and biological function of SETDB2 in TC remain not clarified yet.

Materials and Methods

Database Analysis

UALCAN server (http://ualcan.path.uab.edu/analysis) was used to analyze the transcription levels of SETDB2 expression in TC and the adjacent normal thyroid tissues, and estimate the relationship between SETDB2 expression and gender, age, lymph node metastasis status, cancer stages in thyroid cancer (The Cancer Genome Atlas, TCGA-THCA) dataset. The correlation between SETDB2 expression and Thyroglobulin (TG), TTF1, PAX8 in TC was analyzed with Gene Expression Profiling Interactive Analysis 2 (GEPIA2, http://gepia2.cancer-pku.cn/). Kaplan–Meier analysis of SETDB2 in TCGA-THCA was performed using Kaplan–Meier Plotter (http://kmplot.com/).

Tissues and Ethical Statement

Tissue microarray contained 90 patients with TC who underwent thyroid surgical resection at the first affiliated hospital of Dalian Medical University from January 2013 to February 2021. The diagnosis of all cases were reconfirmed by at least two experienced pathologists in a blinded manner, according to the 2022 WHO Classification of Thyroid Neoplasms [23]. The clinicopathological data of all specimens are shown in Table 1. The project was approved by the Ethics Committee of the first affiliated hospital of Dalian Medical University (PJ-KS-KY-2022-326) and all subjects signed an informed consent.

Clinicopathological characteristic		primary PTC n=58	ATC n=8	recurrent PTC n=16	metastatic PTC n=8
Age/year	≥55	11 (19.0)	7 (87.5)	8 (50.0)	4 (50.0)
	<55	47 (81.0)	1(12.5)	8 (50.0)	4 (50.0)
Gender	Male	17 (29.3)	5 (62.5)	5 (31.3)	3 (37.5)
	Female	41 (70.7)	3 (37.5)	11 (68.7)	5 (62.5)
Tumor size D/cm	≤1	30 (51.7)	0 (0)	-	2 (25.0)
	>1	28 (48.3)	8 (100)	-	6 (75.0)
Multifocality	Yes	10 (17.2)	0 (0)	-	4 (50.0)
	No	48 (82.8)	8 (100)	-	4 (50.0)
Hashimoto's thyroiditis	Yes	4 (6.9%)	-	-	-
	No	54 (93.1%)	-	-	-
Lymph node metastasis	Yes	36 (62.1)	6 (75.0)	-	-
	No	22 (37.9)	2 (25.0)	-	-
Capsule infiltration	Yes	48 (82.8)	4 (50.0)	-	-
	No	10 (17.2)	4 (50.0)	-	-
Vascular involvement	Yes	-	3 (37.5)	-	-
	No	-	5 (62.5)	-	-

Table 1: The clinical characteristics of included patients.

Data are given as number (n), percentage (%)

Cell Culture and Lentivirus Infection

The PTC cell lines BCPAP and ATC cell lines BHT101 were purchased from the National Collection of Authenticated Cell Cultures of the Chinese Academy of Science. Cells were cultured in DMEM medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) (Gibco, USA) at 37°C with 5% CO2. Full length cDNA of SETDB2 was cloned into pLenti-CMV-GFP-Puro and confirmed by DNA sequencing. The plasmid was then transfected into HEK-293T cells, using virus-packing plasmids from Lipo2000 (Invitrogen, USA) according to manufacturer's instructions, to produce the lenti-virus. The culture supernatants were collected at 48h, and added to BCPAP and BHT101 to construct stable cell lines using positive selection by puromycin. Overexpression efficiency was determined by RT-qPCR.

Immunohistochemical Staining

The immunohistochemistry (IHC) was performed according to our previous protocol. In brief, the slides were boiled in EDTA (PH=8.0) buffer for 15min at 100°C. Then, the sections were subjected with 0.3% H_20_2 solution and were incubated overnight at 4° C with primary antibody anti-SETDB2 (#A7391, Abclonal, China), anti-TG (# MAB-0797, Maixin Biotech, China), anti-TTF1 (# MAB-0677, Maixin Biotech, China), anti-PAX8 (# ZM-0468, ZSGB-BIO, China), DAB stained, and hematoxylin re-stained. The breast cancer tissues were used as positive control, and rabbit/mouse IgG were served as negative control according to manufacturer's protocol. The IHC staining sections were evaluated by two senior pathologists who were blinded to clinical data of the patients. The details as follows: ten 400x fields of view were randomly selected. The intensity of staining was scored as 0 (negative), 1(weakly positive), 2 (moderately positive), 3 (strongly positive), and each field was scored according to the percentage of positive cells: 0 (<5% positive cells), 1 (5%-25% positive cells), 2 (26%-50% positive cells), 3 (51%- 75% positive cells) and 4 (>75% positive cells). The final IHC score is calculated by multiplying the score of staining intensity and the score of percentage of positive cells. Theoretically, the scores can range from 0 to 12.

Cell Proliferation and Colony Formation Assays

The proliferation assay was performed using the Cell Counting Kit-8 (CCK-8, Dojindo, Japan). Cells (1.5×10^3) were seed in 96-well plates and cultured overnight. The number of viable cells was quantified for 0, 24h, 48h, 72 h and 96h, the OD_{450} was detected with microplate reader (Epoch, BioTek, USA). For the colony formation assay, cells (at the density of 1500 cells per well) were incubated in complete medium on 6-well plates for 14 days. The medium was replaced every 3 days. Cells colonies were fixed with paraformaldehyde and staining with crystal violet (0.1%) for 15 min.

Reverse Transcription-Quantitative PCR (RT-Qpcr)

Total RNA of PTC cells and ATC cells were extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA synthesis was conducted using First-Strand cDNA Synthesis Kit (Monad, China) and RT-qPCR was performed using MonAmp™ qPCR Mix (Monad, China) as recommended by the manufacturer. GADPH was used as loading control. The primers were synthesized from Sangon Biotech (Shanghai, China) and the sequences were as follows: SETDB2: 5′-CTGCCAATCAAATGTCACTTCC-3′ (Forward) and 5′-CCACGTTTCGTAGACTCCTTCC-3′ (Reverse); TTF-1: 5′-CGCGTTTAGACCAAGGAAC-3′ (Forward) and 5′-GAGTGTGCCCAGAGTGAAG-3′ (Reverse); PAX-8: 5′-AGGTGGTGGAGAAGATTGG-3′ (Forward) and 5′-ATAGGGAGGTTGAATGGTTG-3′ (Reverse); GADPH: 5′-CAACGGATTTGGTCGTATTGG-3′ (Forward) and 5′-TGATGGCAACAATATCCACTTTACC-3′ (Reverse).

Statistical Analysis

The data was processed using GraphPad Prism 9.0 (San Diego, USA) or SPSS (version 26, IBM). Data are presented as mean ±standard deviation (SD). Nonpaired Student's t-test or Mann-Whitney U test was used for two-group comparison. The ANOVA test was used for more than two group comparison. The relationship between SETDB2 and TG, TTF1, PAX8 expression levels using the Pearson correlation coefficient. *P*-value (*P*) < 0. 05 was considered statistically significant difference (**P*<0.05, ***P*<0.01 and ****P*<0.001).

Results

Clinical Characteristics of the Study Population

The clinicopathological characteristics of the patients are summarized in Table 1. In brief, the majority (70.7%) patients were female in the PTC group, and most (81%) were under 55 years; about half of the specimens (51.7%) were less than 1cm. However, 62.5% patients of the ATC group were male, most (87.5%) were older than 55 years, and all were over 1cm.

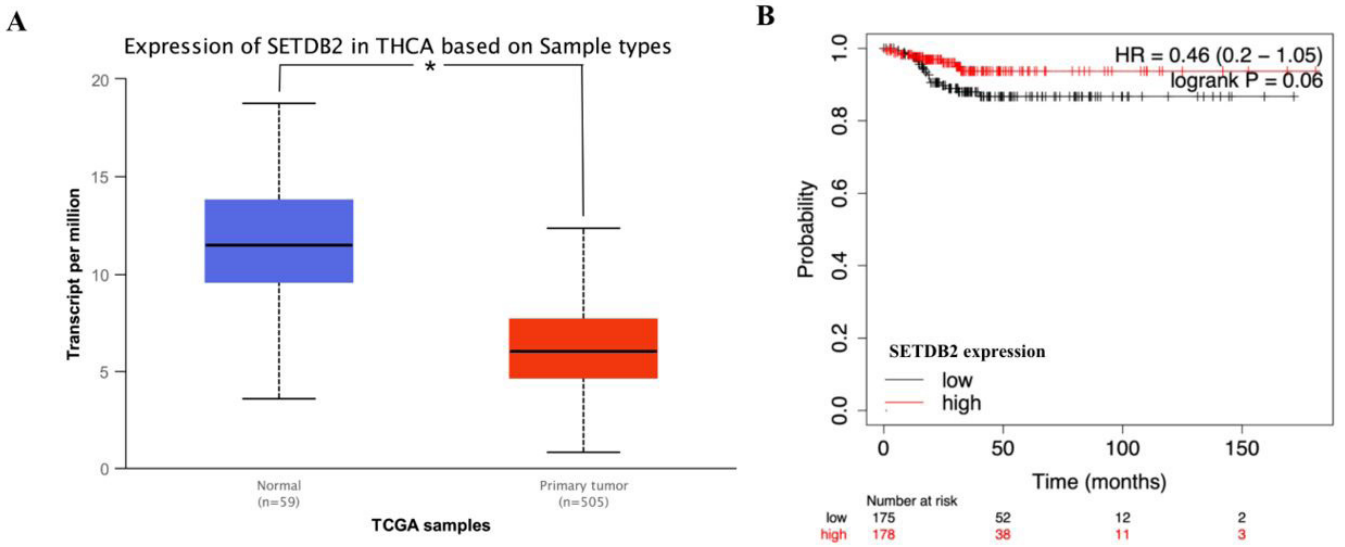
SETDB2 was Differentially Expressed in TC and Adjacent Non- Malignant Tissues

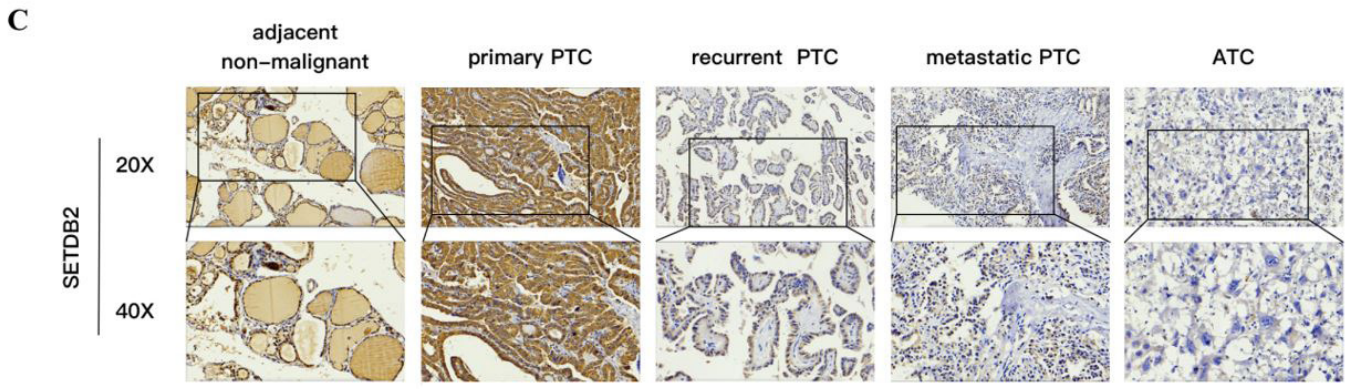
To explore the expression patterns and clinical significance of SETDB2 in TC, we first analyzed the expression of SETDB2 in TC and adjacent normal tissues from TCGA-THCA dataset, and the results showed that the expression of SETDB2 was lower in TC than that in adjacent normal tissues at mRNA level (*P*<0.05, Figure 1A). In addition, according to Kaplan-Meier survival analysis, the recurrence free survival (RFS) was longer in patients with higher expression of SETDB2 than lower expression, however, there was no significant statistical difference (*P*=0.06, Figure 1B).

Next, the expression level of SETDB2 was evaluated by IHC staining in the tissue microarrays and the IHC score were summarized in Table 2. The expression of SETDB2 was significantly reduced in TC compared to adjacent non-tumor tissues. Notably, compared with primary PTC, the expression level of SETDB2 was significantly down-regulated in recurrent PTC (*P*<0.001), metastatic PTC (*P*<0.001) and ATC (*P*<0.001). The IHC staining of SETDB2 is relatively weak in ATC, and showed clear nuclear staining of SETDB2 (Figure 1C, D). Collectively, these results indicated that SETDB2 expression was down-regulated in TC compared to adjacent non-tumor tissues, especially in ATC.

Score	adjacent non-tumor n(%)	primary PTC n(%)	recurrent thyroid cancer n(%)	metastatic thyroid cancer n(%)	ATC n(%)	*P -value*
0	0	0	2 (12.5)	0	2 (25)	<0.01
1	0	0	0	0	0	
2	0	0	4 (25)	0	1 (12.5)	
4	0	0	9 (56.3)	4 (50)	4 (50)	
8	30 (51.7)	25 (43.1)	1 (6.3)	4 (50)	1 (12.5)	
12	28 (48.3)	33 (56.9)	0	0	0	

Table 2: SETDB2 expression in adjacent non-tumor and TC tissues, as assessment by IHC score.
Score grade: 0 (none; <5% positive cells), 1 (weak; 6-25% positive cells), 2 (moderate; 26-50% positive cells), 3 (strong; 51-75% positive cells) or 4 (>75% positive cells)





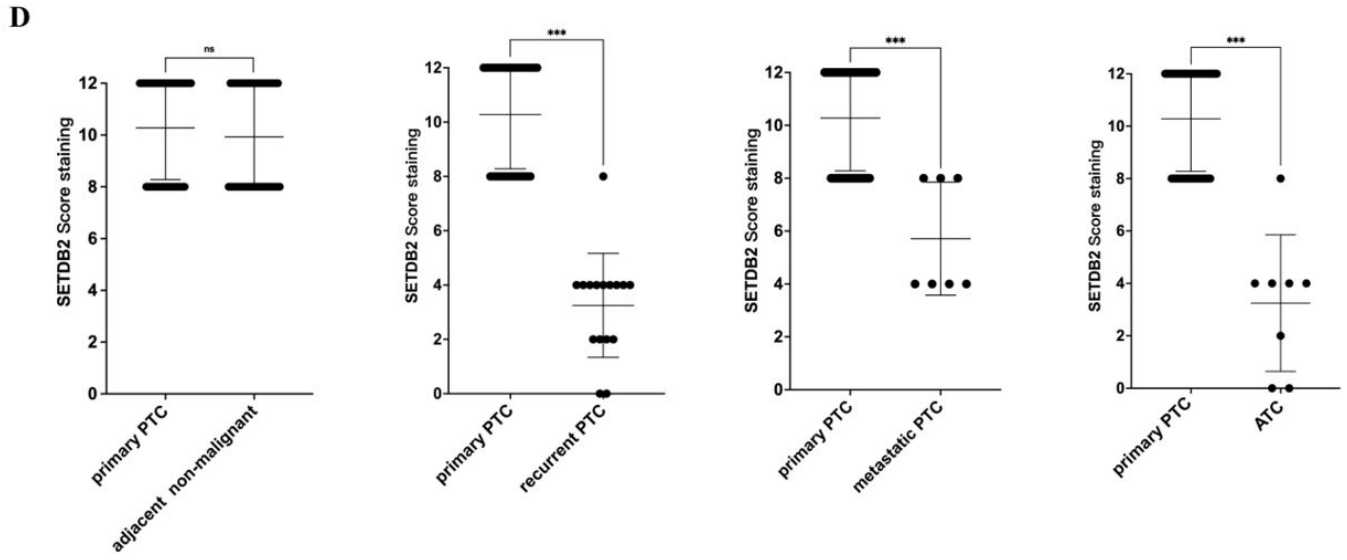


Figure 1: SETDB2 expression patterns in TC and adjacent non-malignant tissues. (**A**) The UALCAN server was used to analyze the expression levels of SETDB2 in TC and adjacent normal tissues from the TCGA-THCA dataset (normal, n=59; thyroid cancer, n=505, **P*<0.05); (**B**) Kaplan-Meier survival curves of prognosis-related SETDB2 expression in the Kaplan-Meier plotter cohort (n=353, *P*=0.06; Log-rank test); (**C**) Representative IHC image showing the expression of SETDB2 in adjacent non-malignant, primary PTC, recurrent PTC, metastatic PTC, ATC tissues (magnification: 20×, 40×); (**D**) Scatter plot of IHC staining score of SETDB2 expression in primary PTC tissues and adjacent non-malignant, recurrent PTC, metastatic PTC, ATC (Nonpaired Student's t-test, **P*<0.05, ****P*<0.001, ns represents no statistical significance)

Relationship Between SETDB2 Expression Levels and Clinicopathological Features

To evaluate the significance of SETDB2 in patients with primary PTC and ATC, we investigated the association between SETDB2 expression levels and the clinicopathological characteristics of patients with primary PTC and ATC. Age was cut-off at 55 years according to the eighth edition of the TNM system of the American Joint Committee on Cancer (AJCC) [24]. The expression of SETDB2 was not associated with gender (*P*=0.670), age (*P*=0.366), tumor size (*P*=0.884), lymph node metastasis (*p*=0.121), capsule infiltration (*P*=0.552) or Hashimoto's thyroiditis (*P*=0.453) in primary PTC group, however, it was significantly correlated with multiplicity (*P*=0.023) which is a high-risk factor of recurrence. There was no association of SETDB2 expression with gender (*P*=0.640), age (*P*=0.263), tumor size (*P*=0.400), lymph node metastasis (*P*=0.108), and capsule infiltration (*P*=0.215) in ATC group (Table 3), the small sample size of ATC may account for the results. Moreover, an analysis of the association between SETDB2 expression and gender (*P*>0.05), age (*P*>0.05), lymph node metastasis status (*P*>0.05) was performed in the TCGA-THCA dataset, and were consistent with our conclusion (Figure 2A-C). Notably, the lower expression of SETDB2 was shown to be associated with the worse status of clinical TNM stages in TCGA (*P*<0.05, Figure 2D).

		primary PTC (n=58)		ATC (n=8)	
		Z	*P*	Z	*P*
Age/year	≥55	-0.903	0.366	-1.119	0.263
	<55				
Gender	Male	-0.426	0.670	-0.468	0.640
	Female				
Tumor size D/cm	≤1	-0.196	0.884	-0.841	0.400
	>1				
Lymph node metastasis	Yes	-1.437	0.121	-1.609	0.108
	No				
Capsule infiltration	Yes	-0.552	0.581	-1.239	0.215/
	No				
Multifocality	Yes	-2.270	0.023*	-	-
	No				
Hashimoto's thyroiditis	Yes	-0.751	0.453	-	-
	No				

Table 3: Clinicopathological correlations of SETDB2 IHC score in PTC and ATC patients.

*$P<0.05$

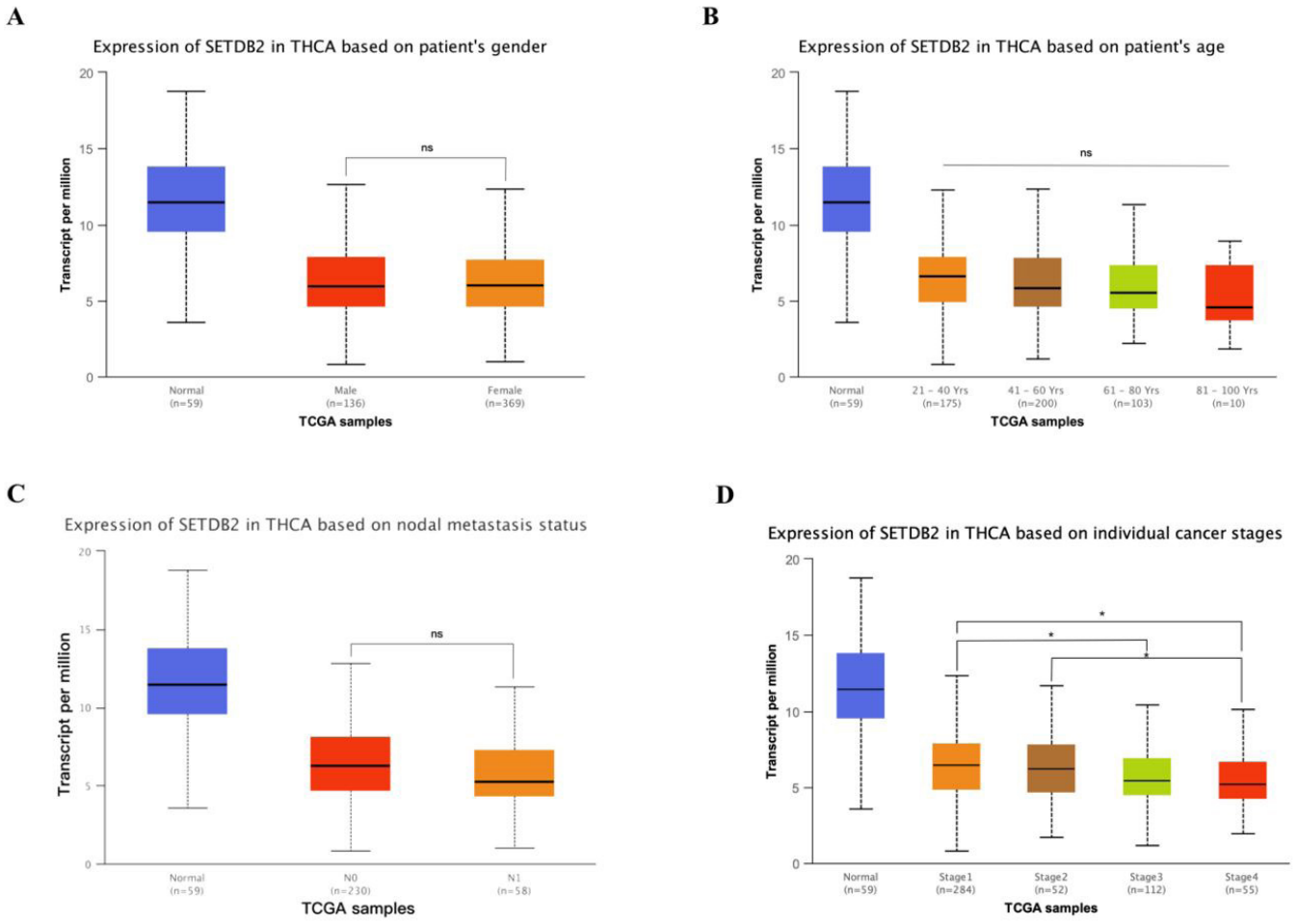


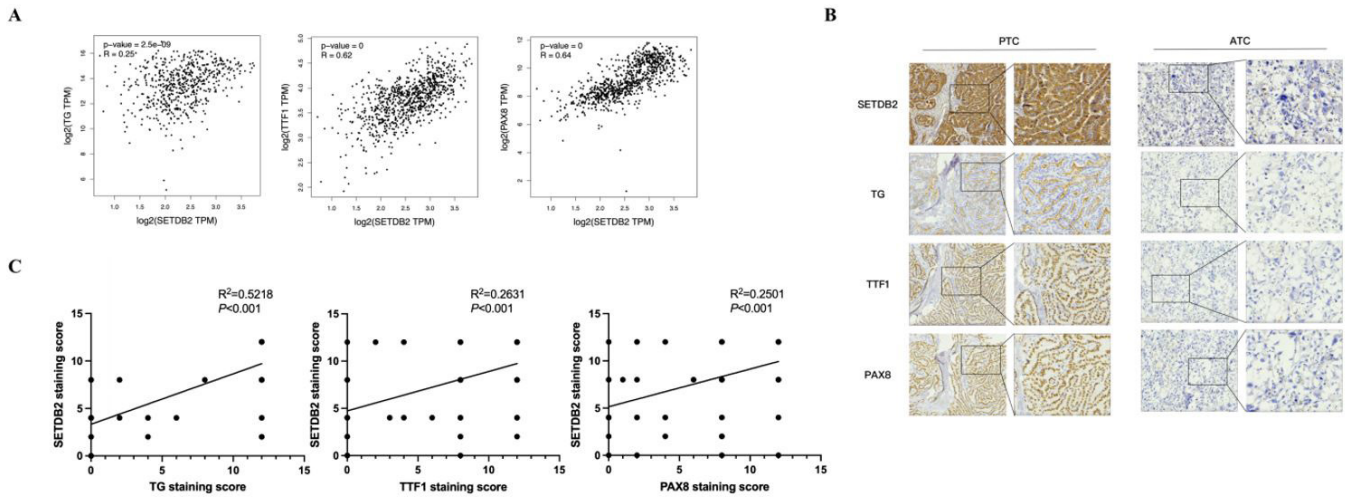
Figure 2: Relationship between SETDB2 expression levels and clinicopathological features in the TCGA-THCA dataset. Expression of SETDB2 in TC based on patient's gender (**A**), patient's age (**B**), nodal metastasis status (**C**), individual cancer stages (**D**) from UALCAN analysis. *$P < 0.05$, ns represents no statistical significance

SETDB2 was Positively Correlated with Thyroid Differentiation Markers and Inhibited Cell Growth

Considering the importance of losing thyroid follicular cell differentiation in TC, we investigate the association between expression of SETDB2 and thyroid differentiation genes (TG, TTF1, PAX8) in the TCGA-THCA dataset. The analysis suggested a positive correlation between the expression of SETDB2 and TG, TTF1, PAX8 at mRNA levels (Figure 3A). In addition, IHC staining also verified the results in our clinical cohort (Figure 3B). As shown in Figure 3C, there was a significant positive correlation between SETDB2 and TG (R^2=0.5218, P<0.001), TTF1 (R^2=0.2631, P<0.001), PAX8 (R^2=0.2501, P<0.001).

In order to further confirm the role of SETDB2 in TC cells, we transfected PTC cell lines BCPAP and ATC cell lines BHT101 with plasmid. Transfection efficiency was confirmed by RT-qPCR (Figure 3D). At mRNA level, both TTF1 and PAX8 were upregulated in BCPAP after SETDB2 overexpression while only TTF1 was upregulated in BHT101, however, the PAX8 level was low in BHT101 with CT value more than 29 in qPCR experiments (Figure 3E, F).

Moreover, the effect of SETDB2 on the proliferative ability of PTC cell lines BCPAP and ATC cell lines BHT101 was assessed by CCK-8 assay and colony formation assay. The result showed that the overexpression of SETDB2 markedly inhibited the cell growth and colony formation capacities in BCPAP and BHT101 cells (Figure 3G-I). Therefore, these finding indicated that SETDB2 was highly correlated with thyroid differentiation markers, which may play a crucial role in the dedifferentiation of TC, and inhibited the proliferation ability of PTC and ATC cell lines in vitro.



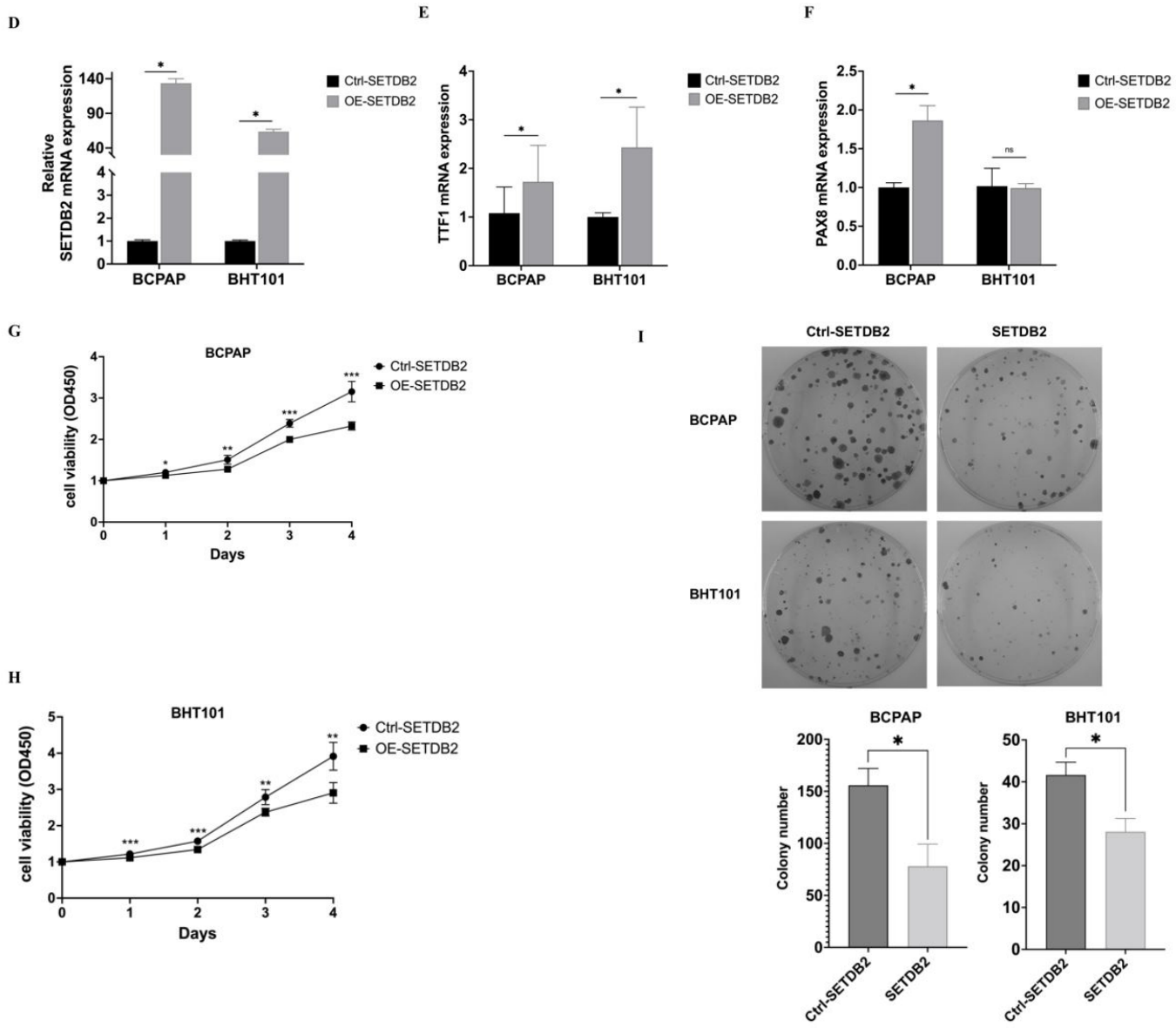


Figure 3: SETDB2 expression was positively associated with TG, TTF1 and PAX8 and inhibited growth of PTC and ATC cells. (**A**) The GEPIA2 server was used to analyze the correlation between SETDB2 expression and TG, TTF1 and PAX8 in TC; (**B**) IHC staining of SETDB2, TG, TTF1 and PAX8 in ATC and PTC tissues; (**C**) Statistical Analysis; (**D-F**) The expression levels of SETDB2, TTF1, PAX8 were detected by RT-PCR after SETDB2 overexpression in BCPAP and BHT101cells; The effects on proliferation ability in BCPAP (**G**) and BHT101 (**H**) cells via CCK-8 assay after SETDB2 overexpression; (**I**) The effects on colony formation ability in BCPAP and BHT101 cells via colony formation assay after SETDB2 overexpression. All data are presented as the mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$

Discussion

ATC represents a major therapeutic challenge in TC treatment, although immunotherapy and targeted therapy have achieved initial success, the survival rate of ATC remains extremely low [6; 25]. It has been shown that dedifferentiation occurs in approximately 5% of patients during TC progression and is usually accompanied by more aggressive growth, metastasis, and loss of iodine uptake, resulting in patients' resistance to conventional treatments as well as radioiodine therapy [26; 27]. However, the exact pathogenesis of ATC is still unclear. Therefore, it is crucial to better understand the underlying mechanisms of ATC tumorigenesis.

The carcinogenesis of ATC includes abnormalities in multiple molecules, such as driver mutations, gene fusions and oncogenic pathway activation [28; 29]. In contrast, epigenetic modifications that do not change the DNA sequence can also regulate transcriptional activation or suppression. A variety of epigenetic dysregulations have been reported in TC [30]. Among the complicated mechanisms, histone methylation is one of the most common types for gene transcription regulation in cancer, which plays a fundamental role in cellular differentiation and development. Notably, EZH2, an H3K27 methyltransferase, has been proved to be associated with dedifferentiation of TC [31; 32].

However, SETDB2, an H3K9 methyltransferase, has not been extensively studied in tumors. Initially, researchers identified that SETDB2 played a key role in early embryonic development of zebrafish [33; 34]. In recent years, studies have established that SETDB2 was involved in lipid metabolism [35], diabetes [36], and atherosclerosis [37]. Some publications have reported that SETDB2 played an oncogenic role in solid tumors and hematologic tumors. In gastric cancer, SETDB2 has been shown to suppress the expression of oncogenes WWOX, CADM1, promoted tumor proliferation and invasion, and was associated with poorer prognosis [19]. In renal tumors, high expression of SETDB2 was associated with metastasis spread [21]. In addition, Lin et al. elucidated that SETDB2 inhibited CDKN2C expression through H3K9me3, which in turn led to the development of acute leukemia [22]. Recent findings by Liu et al. showed that SETDB2 played a crucial role in maintaining breast cancer stem cells through interaction with ANp63a [20].

To our knowledge, this is the first study to explore the role of SETDB2 in TC. We revealed that the expression of SETDB2 was significantly down-regulated in ATC tissues compared to PTC by IHC and its expression levels was positively correlated with TG, TTF1 and PAX8. In addition, PTC cell lines BCPAP and ATC cell lines BHT101 were chosen in this study to conduct experiments in vitro. The results consistent with our hypothesis that SETDB2 could inhibit tumor growth and the expression of TTF1 and PAX8 were upregulated after SETDB2 overexpression in PTC and ATC cell lines at mRNA level. Therefore, our findings provided evidence that SETDB2 might play a critical role in the process of dedifferentiation in TC and may be regarded as a potential therapeutic biomarker.

The present study provides some new findings, however, it still has several limitations. Firstly, the study was limited by the sample size, it is hard to acquire large samples due to the low prevalence of ATC. Consequently, the relationship between SETDB2 expression and the clinicopathological characteristics was not observed in ATC patients. Secondly, this is an in vitro study, our findings need to be verified by in vivo studies. Thirdly, due to the tumor heterogeneity, TC cell lines could not totally mimic the process of carcinogenesis in vivo, this led to some unsatisfactory in vitro results. Furthermore, the commercial ATC cell lines were very limited which is more or less a barrier for ATC research. Lastly, to understand the underlying molecular mechanism that SETDB2 involved in dedifferentiation, further investigations is required.

Conclusion

In summary, our results demonstrated that compared to PTC tissues, SETDB2 was down-regulated in ATC. Moreover, the overexpression of SETDB2 promoted thyroid differentiation markers expression and suppressed tumor growth in PTC and ATC cell lines. Therefore, SETDB2 may plays a key role in the process of dedifferentiation in TC and was expected to be a candidate for therapeutic marker.

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Authors Contributions

Qi Jiang performed the experiments, data collection and analysis, as well as performing the research and drafting the manuscript. Qingzhu Duan and Haifeng Luo analyzed the data, prepared figures and/or tables. Feiliyan Maimaiti and Tianci Shen contributed reagents/materials/analysis tools. Zhikun Lin and Jian Jiang authored or reviewed drafts of the manuscript. Jiangning Gu and Dan Chen conceived and designed the study and revision of the manuscript. All authors revised and approved the manuscript.

Ethics Approval and Consent To Participate

This study was approved by the Ethics Committee of the First Affiliated Hospital of Dalian Medical University of Science and Technology. All experiments were performed in accordance with relevant guidelines and regulations. All patients provided written informed consent prior to the commencement of the study.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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