

RETRACTED ARTICLE: HIF1 α /PD-L1 axis mediates hypoxia-induced cell apoptosis and tumor progression in follicular thyroid carcinoma

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Background: Hypoxia-inducible factor 1 α (HIF1 α) and programmed cell death-1 protein ligand 1 (PD-L1) are implicated in the metastasis and progression processes of multiple cancers. Hypoxia selectively elevates PD-L1 expression via HIF1 α activation in several solid tumors; however, the regulatory effect of HIF1 α on PD-L1 in the pathogenesis of follicular thyroid cancer (FTC) remains unclear. This study aims to investigate the regulatory effect of HIF1 α on PD-L1 and their potential roles in FTC pathogenesis.

Methods: Spearman correlation analysis was performed to clarify the relationships between HIF1 α and PD-L1 expressions and the clinicopathologic characteristics. The expressions of HIF1 α and PD-L1 at mRNA and protein levels were analyzed by qRT-PCR and Western blot. Hypoxia induction and cell transfection were conducted in FTC cells. TUNEL and Annexin V staining were used to detect the cell apoptosis. FTC xenograft tumor models were generated to evaluate the roles of HIF1 α and PD-L1 in vivo.

Results: Here, we found that the expressions of HIF1 α and PD-L1 were significantly increased in FTC tissues and were correlated with the FTC clinicopathologic features, such as the tumor size, T stage, TNM staging, and metastasis. In FTC cells, hypoxia-induced increase of HIF1 α and PD-L1 expression. Knockdown of HIF1 α inhibits hypoxia-induced PD-L1 expression and cells apoptosis. Moreover, inhibition of HIF1 α or PD-L1 significantly delays tumor growth and metastasis in vivo.

Conclusion: Hypoxia could promote FTC progression by upregulating HIF1 α and PD-L1, which could serve as the molecular targets for FTC treatment.

Keywords: follicular thyroid cancer, HIF1 α , PD-L1, hypoxia, apoptosis

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Introduction

Thyroid carcinoma is the most common endocrine malignancy with different types including anaplastic thyroid carcinoma (ATC), well-differentiated thyroid cancer, follicular thyroid cancer (FTC) and papillary thyroid cancer (PTC).¹ The pathological mechanisms underlying FTC are still not fully understood. Therefore, exploration of the molecular mechanism involved in FTC progression is vital for early diagnosis and effective prevention for FTC.

By mediating oncogene activation and loss of tumor suppressors, hypoxia plays a crucial role in tumor cell invasion, metastasis, and progression.² In response to hypoxia situation, hypoxia-inducible factor (HIF) is upregulated to coordinate an appropriated adaptive response.³ HIF is a highly conserved transcription factor comprising a regulatory α subunit (HIF1 α , HIF2 α , or HIF3 α) and a constitutive β

subunit (HIF- β).⁴ It has been reported that hypoxia-induced overexpression of HIF1 α promotes epithelial-mesenchymal transition in FTC.⁵ Moreover, pigment epithelium-derived factor exhibits an antiangiogenesis role in FTC by affecting the HIF1 α /VEGF pathway, eventually inhibits FTC metastasis and progression.⁶ These findings attach much significance to HIF1 α expression on FTC development, while the correlation between HIF1 α expression level and FTC clinicopathologic characteristics and its effect on cell apoptosis remain unclear.

Programmed cell death-1 protein 1 (PD-1) is a checkpoint molecule on T cells and is overexpressed in the tumor environment. PD-L1 is the primary ligand of PD-1.⁷ So far, immune checkpoint inhibitor therapies targeting PD-L1/PD-1 have been shown to be effective in treating various human cancers.⁸ High expression of PD-L1 in tumors is closely associated with cancer immune evasion and often regarded as a negative prognostic factor.⁹ It has been claimed that the expression of PD-L1 and PD-1 was significantly correlated with prognosis of FTC patients, suggesting an effective immunotherapy for FTC treatment FTC by targeting PD-L1/PD-1.⁸ Moreover, PD-L1 overexpression acted as a useful prognostic marker for aggressive FTC and correlated with a great risk of FTC recurrence.^{10,11} In melanoma cell lines, blocking PD-L1 impairs tumor sphere formation and promotes apoptosis of sphere cells.¹² However, the influence of PD-L1 on FTC cell apoptosis has not been clarified yet.

Previous studies have confirmed that hypoxia might selectively elevate PD-L1 expression in some tumors such as pulmonary pleomorphic carcinoma¹³ and oral squamous cell carcinoma.¹⁴ In consideration of the significance of HIF1 α and PD-L1 in FTC development, we initiated this study to investigate the relationship between HIF1 α and PD-L1 expression and FTC clinicopathologic characteristics and the potential functional role in mediating cell apoptosis in FTC.

Materials and methods

Clinical samples

Our study was approved by the Cancer Hospital of University of Chinese Academy of Sciences and performed in accordance with the Helsinki Declaration. All participants signed the written informed consent before the experiments. The clinicopathologic data were collected from 85 FTC patients who were admitted to the Zhejiang Cancer Hospital. Spearman correlation analysis was

performed to analyze the relationships between PD-L1 and HIF1 α expression and the clinicopathologic characteristics including age, sex, tumor size, T stage, lymphocytic thyroiditis, TNM staging, metastasis, psammoma body, and calcification in the stoma. In addition, 85 FTC cancer tissues and the paired cancer-free tissues were taken to detect the expression levels of PD-L1 and HIF1 α .

qRT-PCR

For PD-L1 and HIF1 α expression detection, total RNA was extracted from FTC cancer tissues and the paired cancer-free tissues using the Trizol reagent (Invitrogen) and then was reverse-transcribed with MMLV reverse transcriptase (Invitrogen). RT-PCR was performed on a ViiA7 real-time PCR system using the SYBR Premix Ex Taq™ II Kit (Takara, Dalian, China). Comparison Ct ($2^{-\Delta\Delta Ct}$) method was used to analyze the relative expression, GAPDH was served as an internal control.

Cell culture and cell transfection

FTC H133 and TT cell line were purchased from ATCC (CRL-1803™) and cultured in RPMI-1640 medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 2 mM L-glutamine. The cells were cultured at 37°C in 5% CO₂. siRNA was purchased from the Genepharma (Shanghai, China) and transfected into cells for silencing HIF1 α or PD-L1, respectively. The FTC cells were transfected with siHIF1 α or siPD-L1 at a concentration of 60 nM using Lipofectamine 2000.

Immunofluorescence

The HIF1 α and PD-L1 expressions in FTC cells were determined with immunofluorescence staining. Briefly, FTC cells were fixed with 4% paraformaldehyde for 20 mins at room temperature and then washed with PBS. The fixed cells were permeabilized with 0.5% Triton X-100 for 10 mins at room temperature and washed with PBS. For blocking, 5% BSA was added onto cells and maintained for about 1 h. After 5% BSA removing, the primary antibodies (1:500, Abcam, Camb, UK) against HIF1 α and PD-L1r were added and incubated overnight at 4°C, respectively. Subsequently, cells were incubated with the Alexa fluor 647-conjugated secondary antibody for 1 hr at room temperature, followed by nuclear staining with DAPI (Sigma-Aldrich, St. Louis, USA). Finally, visualization was completed under a fluorescent microscope (Leica, Germany).

Western blot

Total protein extracted from the FTC cells was quantified using a bicinchoninic acid protein assay kit (Beyotime, Shanghai, China), followed by Western blot analysis. A 10 µg of protein was subjected to 10% SDS-PAGE and then transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% BSA in TBS containing 0.1% Tween-20 for 1 hr at room temperature prior to incubation with primary antibodies (anti-HIF1α, anti-PD-L1, and anti-β-actin, Abcam) at 4°C overnight. After washing with PBS, the membranes were incubated with secondary antibody for 1 hr at room temperature and developed with chemiluminescence ECL reagent. Protein bands were quantified using NIH ImageJ software.

Hypoxia induction

For hypoxia induction, FTC cells were maintained in a sealed hypoxic incubator with a mixture of 1% O₂, 5% CO₂, and 94% N₂ at 37°C for 12 hrs⁵. Cells in the normoxia group were cultured under normoxic conditions (21% O₂, 5% CO₂, and 74% N₂) at 37°C for equivalent periods.

TUNEL staining

TUNEL staining assay was performed to determine the cell apoptosis after hypoxia treatment. After hypoxia induction, the FTC cells were dehydrated with ethanol and incubated with the TUNEL reaction mixture at 37°C for 1 hr. Subsequently, cells were washed with PBS to remove the residual liquid. Finally, the TUNEL-positive cells were quantified by averaging five different fields under a light microscope system at 400× magnifications in a blinded manner.

Flow cytometry

Cell apoptosis was detected using flow cytometry with an Apoptosis Detection Kit (Beyotime, Shanghai, China). Briefly, cells were seeded into 24-well plates with 10⁴ cells/well. After 24-hr culture, cells were harvested and stained with 5 µL fluorescein isothiocyanate-conjugated Annexin V at dark for 30 mins according to the manufacturer's instructions. The percentage of apoptotic cells was analyzed using a FACSCalibur flow cytometer.

Establishment of FTC xenograft tumor models

For establishment of FTC xenograft tumor model in mice, FTC cells transfected with the control lentiviral vector or shHIF1α or shPD-L1 were injected into the 6-week-old

male nude mice through the right thyroid lobe. After 28 days, the mice were euthanized for tumor tissues collection, and the tumor volume was detected. All animal experiments were performed according to the guidelines for the care and use of laboratory animals and were approved by the Cancer Hospital of University of Chinese Academy of Sciences. The harvested tumor tissues were used for H&E staining to evaluate the tumor metastasis.

Immunohistochemistry

The expression of proliferating cell nuclear antigen (PCNA) in tumor tissues was examined by immunohistochemistry staining using the streptavidin-biotin-HRP technique. After fixation in 10% buffered formalin and embedding in paraffin, the tissues were deparaffinized in xylene and dehydrated in ethanol, and then blocked with 0.2% H₂O₂ for 15 mins. After washing with PBS and blocking with 5% BSA, the primary antibody against PCNA was added and incubated with tissues overnight at 4°C, followed by the incubation with the second goat anti-rabbit IgG for 1 hr at room temperature. After washing with the PBST, color was developed with the 3,3'-diaminobenzidine by maintaining for 20 min, and a microscope was used for counting at 200× magnifications.

Statistical analysis

Three independent experiments were performed and all data were expressed as mean ± SD. SPSS 21.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Student's *t*-test was used for comparisons between two groups and one-way ANOVA was used for more groups. A value of *p*<0.05 was considered significant.

Results

Correlation between PD-L1 and HIF1α expression and clinicopathologic features

First, with the clinicopathologic data of 85 enrolled FTC patients, we analyzed the relationships between PD-L1 and HIF1α expression and clinicopathologic characteristics, including age, sex, tumor size, T stage, lymphocytic thyroiditis, TNM staging, metastasis, psammoma body, and calcification in stoma. Table 1 demonstrates that PD-L1 expression is correlated with the tumor size (*p*=0.01), T stage (*p*=0.03), TNM staging (*p*=0.005), and metastasis (*p*<0.001); while HIF1α expression is correlated with TNM staging (*p*=0.002), metastasis (*p*<0.001), and PD-L1 expression (*p*<0.001). The results highlighted that PD-L1

Table 1 The relationships between PD-L1 and HIF1 α expression and clinicopathologic characteristics of 85 thyroid carcinoma patients

Variables	No.	PD-L1		p	HIF1 α		p
		Positive	Negative		Positive	Negative	
Total	85	57	28		64	21	
Age (years)				0.885			0.856
<45	48	32	16		37	11	
≥ 45	37	25	12		27	10	
Sex				0.159			0.880
Male	32	18	14		26	6	
Female	53	39	14		41	12	
Tumor size (mm ³)				0.010*			0.055
≥ 40	57	44	13		47	10	
<40	28	13	15		17	11	
T Stage				0.030*			0.084
T1 and T2	49	38	11		33	16	
T3	36	19	17		31	5	
Lymphocytic thyroiditis				0.219			0.051
Yes	39	23	16		21	14	
No	46	34	12		39	7	
TNM				0.005**			0.002**
I + II	47	38	9		42	5	
III+IV	38	19	19		22	16	
Metastasis				0.000***			0.000***
Yes	35	15	20		18	17	
No	50	42	8		46	4	
Psammoma body				0.914			0.268
Yes	12	5	7		7	5	
No	73	52	21		57	16	
Calcification in stoma				0.442			0.116
Yes	43	31	12		36	7	
No	42	26	16		28	14	
PD-L1							0.000***
Positive	67	59	8		59	8	
Negative	18	5	13		5	13	

Note: *p<0.05, **p<0.01, ***p<0.001.

Abbreviation: HIF-1 α , hypoxia-inducible factor 1 α ; PD-L1, programmed cell death-1 protein ligand 1.

and HIF1 α expressions were closely correlated with the FTC clinicopathologic features, suggesting the potential role in FTC progression.

Increased expressions of HIF1 α and PD-L1 in FTC tissue

In order to reveal the potential impact of HIF1 α and PD-L1 on FTC progression, we analyzed their expression

levels in FTC tissues and their corresponded cancer-free tissues. As is shown in [Figure 1A](#), HIF1 α was notably upregulated in FTC tissues compared with that in the cancer-free tissues. Similarly, PD-L1 expression was significantly higher in FTC tissues than that in the cancer-free tissues ([Figure 1B](#)). Spearman correlation analysis indicated that the expression level of HIF1 α was positively correlated with that of PD-L1 in FTC tissues ([Figure 1C](#)), suggesting the potential interaction between them.

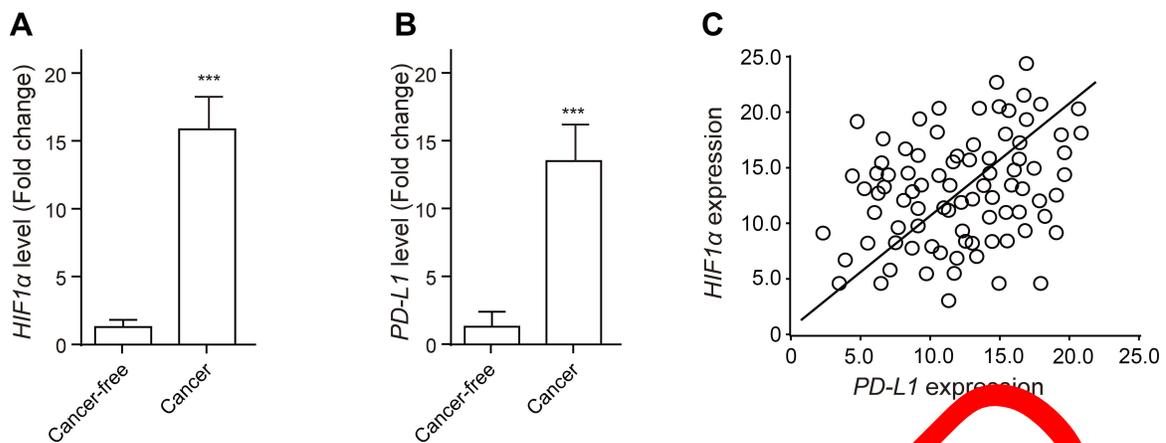


Figure 1 Increased expressions of HIF1α and PD-L1 in FTC tissue. The expression levels of HIF1α (**A**) and PD-L1 (**B**) in FTC tissue and in cancer-free tissue were analyzed using qRT-PCR. (**C**) Spearman correlation analysis was performed to clarify the relationships between HIF1α and PD-L1 expression. Data presented as mean ± SD, ***p<0.001. **Abbreviations:** HIF-1α, hypoxia-inducible factor 1α; PD-L1, programmed cell death-1 protein ligand 1.

Upregulation of HIF1α and PD-L1 upon hypoxia induction in FTC cells

To clarify the influence of hypoxia induction on HIF1α and PD-L1 expression, the FTC cells lines TT and

FTC-133 were used and cultured under normoxia or hypoxia condition, respectively. The results of immunofluorescence staining demonstrated that hypoxia induction significantly promoted the expression of

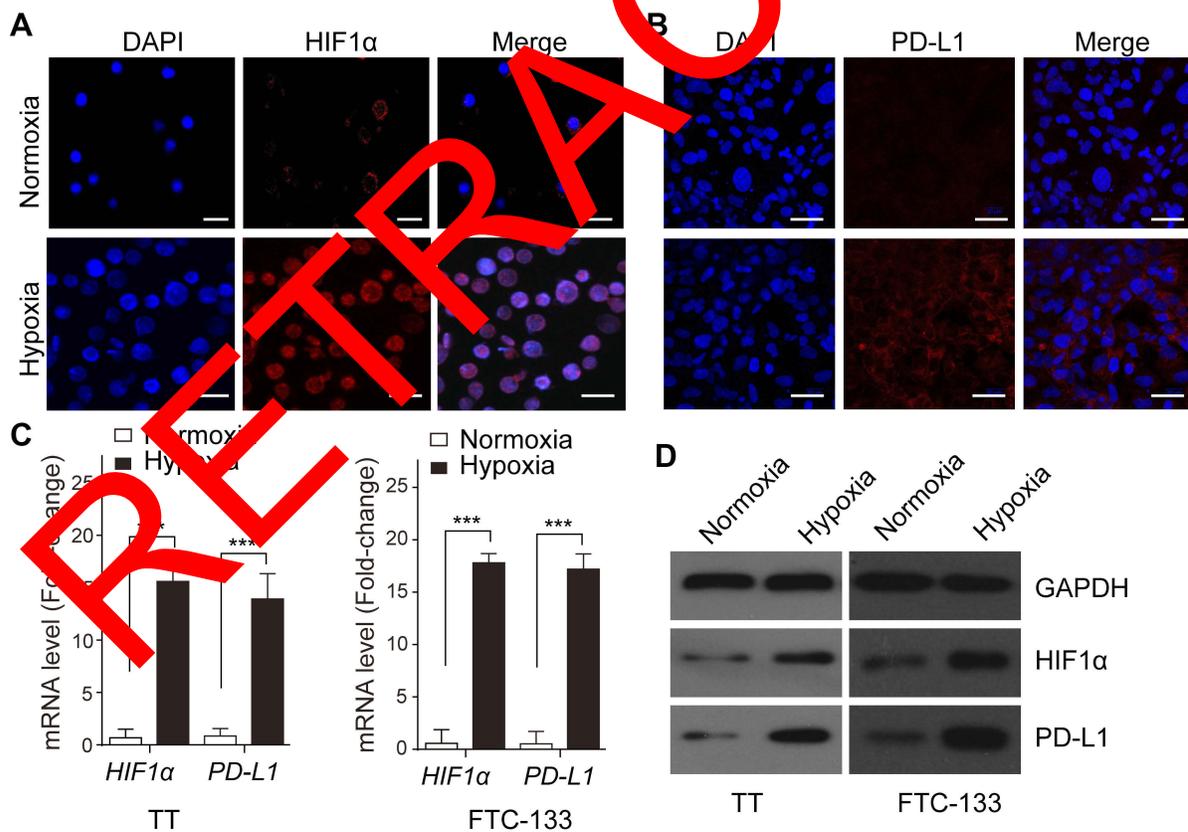


Figure 2 Upregulation of HIF1α and PD-L1 upon hypoxia induction in FTC cells. The expressions of HIF1α (**A**) and PD-L1 (**B**) in TT cells were determined by immunofluorescence staining. (**C**) The expressions of HIF1α and PD-L1 mRNA in TT and FTC-133 cells were examined by qRT-PCR. (**D**) The levels of HIF1α and PD-L1 protein in TT and FTC-133 cells were analyzed by Western blot. Data presented as mean ± SD, ***p<0.001. Scale bar=20 μm.

Abbreviations: HIF-1α, hypoxia-inducible factor 1α; PD-L1, programmed cell death-1 protein ligand 1.

HIF1 α (Figure 2A) and PD-L1 (Figure 2B) in FTC cells. Moreover, hypoxia induction treatment largely increased the expression of HIF1 α and PD-L1 at mRNA (Figure 2C) and protein levels (Figure 2D) in TT and FTC-133, respectively. These data revealed that the expression of HIF1 α and PD-L1 in FTC cells was augmented under hypoxia condition.

HIF1 α induces PD-L1 expression in FTC cells under hypoxia condition

To investigate the impact of HIF1 α on PD-L1 expression in FTC cells treated with hypoxia induction, HIF1 α was silenced by transfecting with siRNA

targeting HIF1 α , and the siControl served as its negative control. As is shown in Figure 3A, HIF1 α expression was largely downregulated after siHIF1 α transfection, and the expression of PD-L1 was also suppressed in cells transfected with siHIF1 α (Figure 3B). The increased expression of HIF1 α and PD-L1 induced by hypoxia was significantly reduced by siHIF1 α transfection, while no significant difference was noted between siControl and siHIF1 α groups in FTC cells under normoxia condition. Silence of HIF1 α also led to decreased protein level of HIF1 α and PD-L1 in FTC cells under hypoxia condition (Figure 3C,D). It indicated that HIF1 α mediated PD-L1 expression under hypoxia condition in FTC cells.

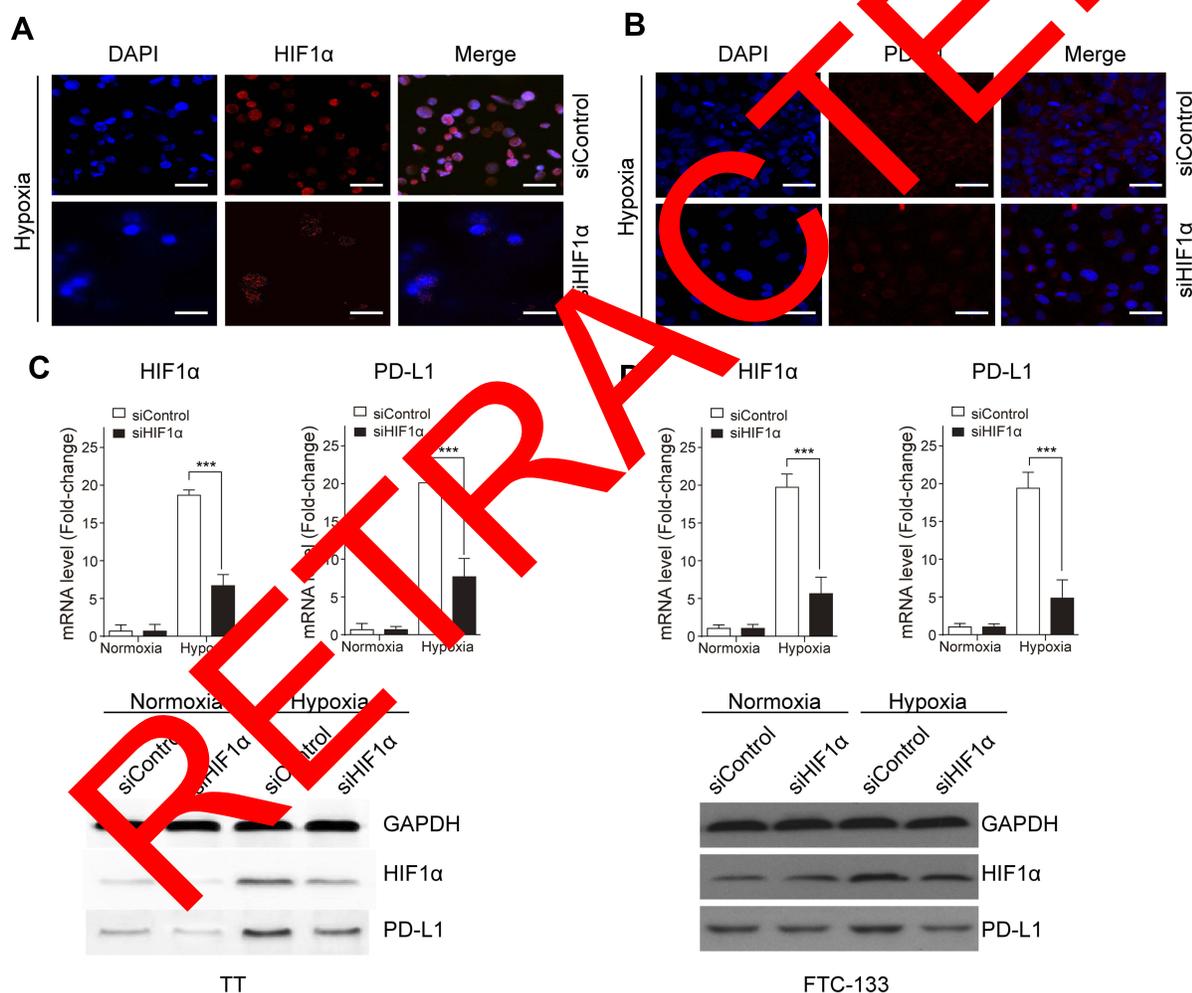


Figure 3 HIF1 α induces PD-L1 expression in FTC cells under hypoxia condition. FTC cells were transfected with siHIF1 α or siPD-L1 or the negative control (siControl). The expressions of HIF1 α (A) and PD-L1 (B) in TT cells were determined by immunofluorescence staining. (C) The mRNA and protein expressions of HIF1 α and PD-L1 in TT cells were determined by qRT-PCR and Western blot. (D) The mRNA and protein expressions of HIF1 α and PD-L1 in FTC-133 cells were determined by qRT-PCR and Western blot. Data presented as mean \pm SD, *** p <0.001. Scale bar=20 μ m.

Abbreviations: HIF-1 α , hypoxia-inducible factor 1 α ; PD-L1, programmed cell death-1 protein ligand 1.

HIF1 α /PD-L1 signaling mediates FTC cell apoptosis

To investigate the functional role of HIF1 α and PD-L1 in thyroid carcinoma, we firstly observed the apoptotic changes in siPD-L1 FTC cells or siHIF1 α FTC cells under the normoxia or hypoxia condition, respectively, and detected by TUNEL and Annexin V staining assay. Knockdown of HIF1 α or PD-L1 promoted hypoxia-induced cell apoptosis in TT and FTC-133 cells (Figure 4). It indicated that HIF1 α and

PD-L1 had a regulatory role in FTC cells survival under hypoxia condition.

Inhibition of HIF1A and PD-L1 delays tumor growth and metastasis in vivo

Next, we investigated the influence of HIF1 α and PD-L1 on tumor growth in vivo, and FTC cells were transfected with shPD-L1 or shHIF1 α through lentiviral vector prior to injection into mice to construct the xenograft tumor

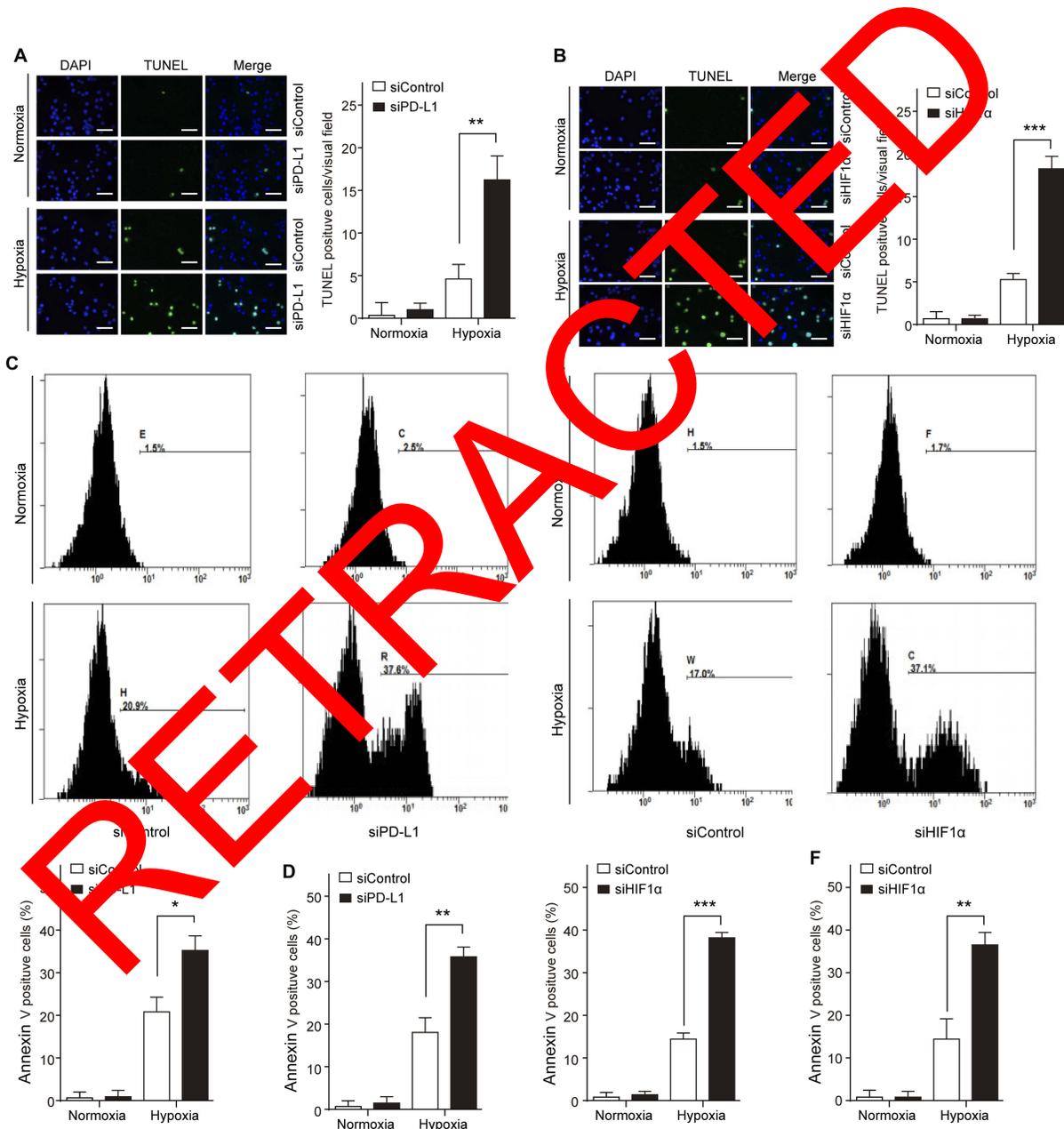


Figure 4 HIF1 α /PD-L1 signaling mediates FTC cell apoptosis. TT and FTC-133 cells were transfected with siHIF1 α or siPD-L1 or the negative control (siControl). Cell apoptosis was determined by TUNEL staining in TT (A) and FTC-133 (B) or by Annexin V staining in TT (C, D) and FTC-133 (E, F). Data presented as mean \pm SD, * p <0.05, ** p <0.01, *** p <0.001. Scale bar=20 μ m.

Abbreviations: HIF-1 α , hypoxia-inducible factor 1 α ; PD-L1, programmed cell death-1 protein ligand 1.

models. The tumor volume was evaluated at 6, 12, 18, and 24 days following injection. It was illustrated that tumor growth was restrained after HIF1 α or PD-L1 knockdown (Figure 5A). Meanwhile, the tumor metastasis was remarkably reduced after HIF1 α or PD-L1 knockdown (Figure 5B), and the positive cells of PCNA staining were significantly reduced after HIF1 α or PD-L1 silence (Figure 5C). Taken together, we concluded that inhibition of HIF1 α /PD-L1 signaling delayed tumor growth and metastasis *in vivo*.

Discussion

In the current study, we demonstrated that PD-L1 expression in FTC is correlated with the clinicopathologic features, including the tumor size, T stage, TNM staging, and metastasis, while HIF1 α expression is correlated with

TNM staging and metastasis. Moreover, HIF1 α expression in FTC is positively correlated with PD-L1 expression. It was noted that the expression of HIF1 α and PD-L1 is clearly upregulated in FTC tissue and in FTC cells induced by hypoxia. In FTC cells treated with hypoxia, HIF1 α upregulates PD-L1 expression, which contributes to suppressing cell apoptosis. In the FTC xenograft tumor models, we verified that downregulation of HIF1 α and PD-L1 delays FTC growth and metastasis. This study identifies PD-L1 as a downstream molecular of HIF1 α and explains the mechanism of antiapoptosis under hypoxia, highlighting the role of HIF1 α in FTC growth, metastasis, and progression.

Due to the rapid proliferation of tumor cells and the abnormality of tumor vessels in function and structure, the hypoxic areas widely exist in solid tumors. By transcriptionally activating diverse target genes, HIF functions as a

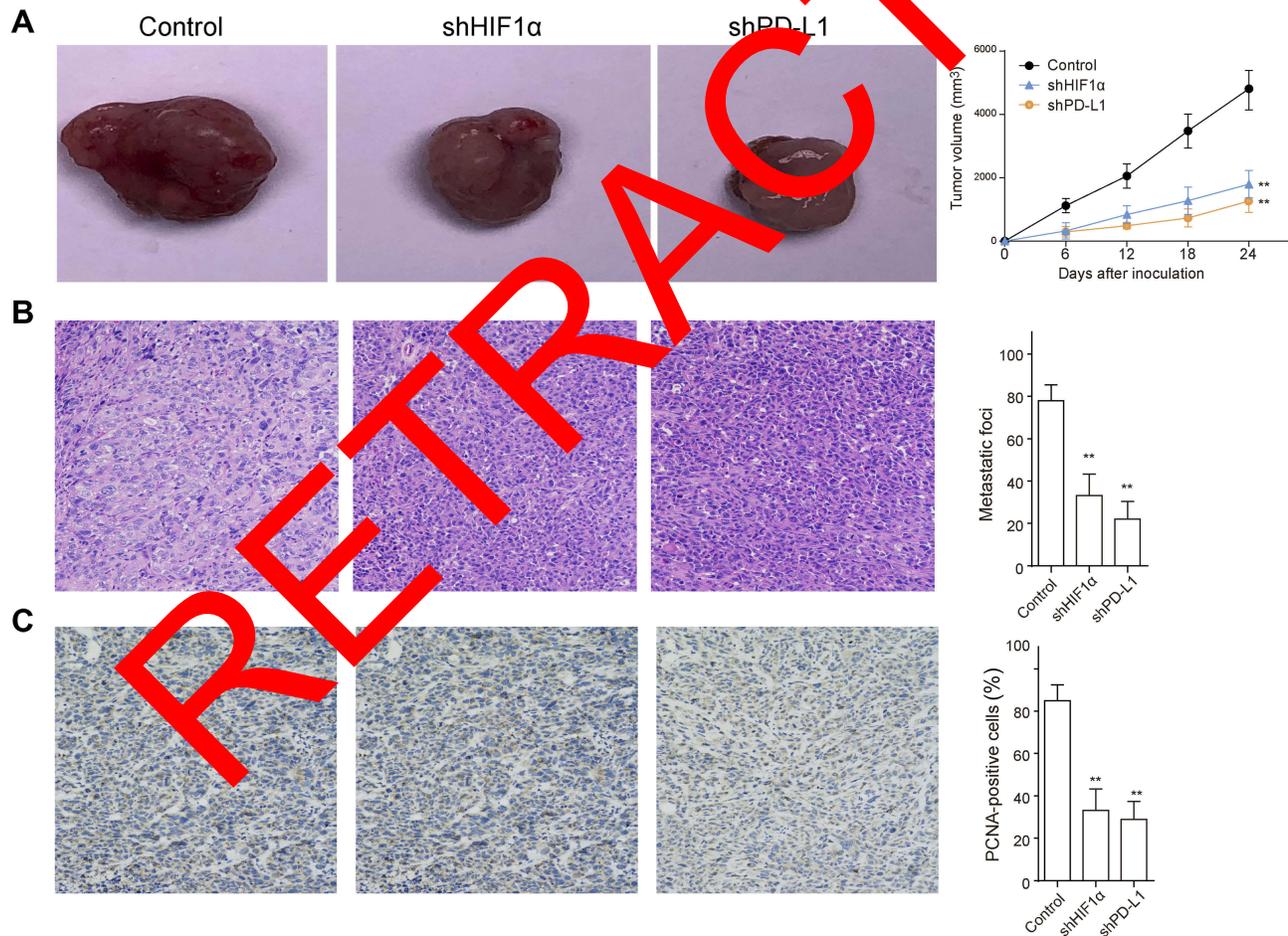


Figure 5 Inhibition of HIF1 α and PD-L1 delays tumor growth and metastasis *in vivo*. FTC cells were transfected with shPD-L1 or shHIF1 α through lentiviral vector were injected into mice to construct the xenograft tumor models. (A) The tumor volume of mice was detected at different time points (0, 6, 12, 18, and 24 days) following injection. (B) The harvested tumor tissues were used for HE staining to evaluate the tumor metastasis. (C) The expression of proliferating cell nuclear antigen (PCNA) in tumor tissues was examined by immunohistochemistry staining. Data presented as mean \pm SD, ** p <0.01. **Abbreviations:** HIF-1 α , hypoxia-inducible factor 1 α ; PD-L1, programmed cell death-1 protein ligand 1.

major adaptive mechanism in tumor growth response to a hypoxic microenvironment, occupying a pivotal position in tumorigenesis.^{17,18} In African breast cancer, high HIF1 α expression is a significant marker of poor prognosis.¹⁹ Via regulating noncoding RNAs, HIF1 α highly contributes to breast cancer metastasis.²⁰ In this study, we firstly analyze the correlation between HIF1 α and FTC clinicopathologic features and revealed that HIF1 α expression is correlated with TNM staging and metastasis. We further indicated that in FTC cells induced by hypoxia, increased HIF1 α upregulates PD-L1 expression to promote cell apoptosis. In addition, our study certifies that interference of HIF1 α delays FTC growth and metastasis in mice model. This study identifies HIF1 α as a key regulator of FTC growth, metastasis, and development, suggesting a promising therapeutic target for FTC.

The previous study has claimed that mortalin-mediated and ERK-controlled targeting HIF1 α to mitochondria confers resistance to apoptosis under hypoxia, implying the antiapoptotic function of HIF1 α that may act as an early protective mechanism upon oxygen limitation.²¹ By increasing expression of apoptosis-related genes, HIF1 α usually exhibits a carcinogenic effect in tumor growth, such as in human salivary adenoid cystic carcinoma.²² The antiapoptotic effect of HIF1 α as well as its indicative function of poor prognosis, has been confirmed in tumors,^{12,23,24} offering a novel insight into developing effective targeted therapies. In the present study, we, for the first time, identified PD-L1 as a downstream target of HIF1 α in repressing apoptosis of FTC under hypoxia. Upon hypoxia, the PD-L1 level was increased by HIF1 α , thereby promoting cell survival. Above all, our data illustrated that interference of PD-L1 in xenograft tumor model markedly controls FTC growth and metastasis. These findings highlight a novel idea to restrain FTC growth by modulating PD-L1 expression.

In summary, our study illuminates that HIF1 α and PD-L1 are increased in FTC. Inhibition of HIF1 α increased PD-L1 expression promotes cell apoptosis, thereby inhibiting cell survival and tumor growth that could be specifically targeted to treat FTC.

Acknowledgment

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Disclosure

The authors declare no conflicts of interest in this work.

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