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ORIGINAL RESEARCH ARTICLE

Tetramethyl thyroxine promotes bladder cancer development by regulating the expression of integrin αV, VEGF, and TP53

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Abstract

Bladder cancer (BC) is the most prevalent malignancy of the genitourinary system, exhibiting the highest morbidity and mortality rates among cancers in this category. Tetramethyl thyroxine (T4) has been recognized to promote the proliferation of various cancer cells. However, the possible effect and underlying mechanisms of T4 on the onset and progression of BC remain to be fully elucidated. Our research demonstrated that T4 significantly promoted the proliferation and migration of EJ-1 and T24 cells. The proliferation of T24 and EJ-1 cells increased by 5 – 28.3% and 4.7 – 18.7%, respectively. Similarly, the scratch healing rates of T24 and EJ-1 cells increased by 9.27 – 41.01% and 11.47 – 35.8%, respectively. In addition, apoptosis of T24 and EJ-1 cells was also significantly reduced after T4 treatment. Furthermore, *in vivo* xenograft tumor model further corroborated that T4 facilitated the growth of EJ-1 cell-derived tumors. Our findings indicated that T4 promoted tumor angiogenesis and cell proliferation by upregulating its receptor integrin αV and vascular endothelial growth factor, while simultaneously suppressed the expression of the tumor suppressor protein TP53. Collectively, our research has determined the tumor-promoting effect and molecular mechanism of T4 on BC through cell and animal models. In the future, by further expanding the sample size and pre-clinical design, it is expected to provide new theoretical foundations and potential targets for the prevention, diagnosis, and treatment of BC.

Keywords: Bladder cancer; Tetramethyl thyroxine; Proliferation; Migration; Integrin αV

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1. Introduction

Bladder cancer (BC), as one of the top ten most prevalent cancers globally, exhibits the highest incidence and mortality rates among urogenital system tumors. According to statistics from the International Agency for Research on Cancer, BC has an incidence rate of 3.1%, ranking ninth globally. In 2022, it is estimated that there will be approximately 614,000 new cases and 220,000 deaths attributable to BC, with the incidence rate significantly higher in men compared to women.[1]

Surgical resection, neoadjuvant chemotherapy, and immunotherapy are common treatments for BC. Although these methods have improved survival rates to a certain extent, high recurrence rates and drug resistance still pose a dilemma in the treatment of BC. The factors contributing to the development of cancer are numerous and complex. They can be broadly categorized into two groups: internal factors, which include mutations in tumor suppressor genes and abnormalities in the immune system, and external factors, which encompass influences from the living environment and unhealthy lifestyle choices. In addition, excessive activation of hormones can also lead to tumor development. For instance, estrogen regulates cell growth and proliferation by binding to estrogen receptors in breast cells,[2] while testosterone and androgens perform a similar function by binding to androgen receptors in prostate cells.[3] Together, these hormones regulate processes such as cell proliferation, growth, and metastasis. They primarily affect genes and proteins associated with proliferation and differentiation in cells through their respective receptors. The main causative factors of BC include chronic infections, pathogens, and other diseases. Furthermore, the development of BC is also linked to hormonal factors, with sex hormones, particularly estrogen and progesterone, being frequently reported in current literature. Croft *et al*. found that the expression of the estrogen receptor ERβ is elevated in BC tissues, with its expression level positively correlated with pathological grade.[4] Ding *et al*. found that estrogen promotes the malignant behavior of BC by inducing the enhancer RNA-P2RY2e.[5] In addition, Wolpert *et al*. reported that the incidence of BC is significantly reduced in parous women and those taking oral contraceptives.[6] However, the relationship and molecular mechanisms linking progesterone to the development of BC remain unclear.

Thyroid hormones, which include T4 and triiodothyronine (T3), are critical regulators of various physiological processes. T4 is also known as thyroxine. Thyroid hormones exert their effects by binding to integrin receptor sites, thereby regulating the expression of target genes. They play a significant role in activating cell growth, inhibiting apoptosis, and stimulating angiogenesis during the carcinogenesis process. Furthermore, a correlation has been observed between elevated thyroid hormone levels, reduced thyroid-stimulating hormone (TSH) levels, and an increased risk of cancer.[7] As a thyroid hormone, T4 has been shown in multiple clinical trials to be associated with the development and poor prognosis of breast cancer, prostate cancer, lung cancer, acute leukemia, and other cancers,[7] but most of these studies stop at analyzing clinical data. An analysis based on the UK Biobank and FinnGen databases found that there was a significant positive correlation between thyroid cancer (THCA) and BC (odds ratio = 1.140; 95% confidence interval, 1.072 – 1.212; $p<0.001$). Correlation analysis showed that THCA may increase the risk of secondary BC by increasing the infiltration of N2 neutrophils. The incidence of THCA in patients with hyperthyroidism is significantly higher than that in ordinary patients.[8] A study on patients with metastatic urothelial carcinoma receiving immunotherapy found that the group with a low free T3/free T4 ratio had shorter progression-free survival and overall survival, which was associated with a poorer prognosis. Moreover, this ratio was independent of other prognostic factors.[9] It indicated a relationship between thyroid hormone and bladder development. However, further mechanistic analysis has not been conducted. Our study aims to reveal the effects of T4 on BC cell proliferation, cell migration, apoptosis, and tumor formation by attempting to investigate its underlying mechanisms.

2. Materials and methods

2.1. Cell lines and reagents

Tetramethyl thyroxine (T4) was purchased from Shanghai Titan Technology Co., Ltd. (HPLC 98.0%, China), dissolved in 0.05M NaOH solution, and stored at –20°C. We selected T4 concentrations based on prior literature linking T4 to cancer progression and on initial pilot experiments.[10] Human BC cell lines (T24, CL-0227, and EJ-1 CL-0274) were kindly provided by Procell Life Science and Technology Co., Ltd and were cultured in RPMI 1,640 medium (HyClone, USA) with 10% fetal bovine serum (Gibco, USA) and 1% PS (Gibco, USA) at 37°C in 5% CO_2. When the cell density had been >80%, discard the culture medium and add 1 ml of trypsin for digestion. After 2 min to separate the cells from the 10 cm culture dish. The cells were passaged at a ratio of 1:2/1:3, once every 2 – 3 days.

2.2. Cell viability by cell counting kit-8 (CCK-8) assay

T24 and EJ-1 cells were inoculated at 5,000 per well in 96 plates and diluted T4 to a range of 0 – 100 nM for treatment. After 24 h and 48 h, drug-containing medium was aspirated. Subsequently, RPMI 1,640 medium containing 10% CCK-8 solution was added to each well and incubated in a 5% CO_2 incubator for 2 h. We performed three biological replicates during the experiment. The absorbance of each well was measured at 450 nm using a Multiskan™ FC microtiter plate instrument (Thermo Fisher Scientific, USA).

2.3. Flow cytometry detection of cell apoptosis

1.5×10^4 T24 and EJ-1 cells were seeded in a 6-well plate, and cells were collected after treatment with T4 at different concentrations (0 nM, 10 nM, 100 nM) for 48 h. Annexin

V-FITC kit (Sigma-Aldrich, USA) was used according to the instructions. The cells were mixed with 5 μL of Annexin V-FITC and 10 μL PI and incubated and stained in the dark for about 15 min. After staining, the solution was transferred to a flow cytometry tube for detection within 1 h, and the effect of T4 treatment on cell apoptosis was evaluated using FlowJo V10 software (BD Biosciences, USA). Three independent biological replications of this experiment were performed.

2.4. Determination of cell migration rate by cell scratch assay

2×10^4 T24 and EJ-1 cells were seeded in equal amounts in a 24-well plate and cultured. After the cells adhered, a 200 μL pipette tip was used to scratch the well plate covered with cells, and then it was washed 2 – 3 times with PBS to wash away the suspended cells. The migration of T24 and EJ-1 cells was observed and recorded using microscopy before the addition of T4 treatment and at 24 h and 48 h post-T4 treatment. Image J software was used to calculate and analyze the effect of T4 treatment on cell migration rate.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

First, treat T24 and EJ-1 with T4 and collect the cells, then use The RNAprep pure cell kit (DP430, Tiangen, China) to extract total RNA. FastKing RT Kit (KR116-02, Tiangen, China) was used to reverse transcribe cDNA and then analyze it quantitatively using Real-Time PCR System (QuantStudio1-A40425, Thermo Fisher Scientific, USA) and PowerUp SYBR Green Master Mix (01000439, Thermo Fisher Scientific, USA) for quantitative analysis. *GAPDH* was selected as the internal reference gene, and expression changes were analyzed computationally using the δδCT algorithm. Primers were purchased from Tsingke Biotechnology Co., Ltd. (China), and details are shown in Table 1.

2.6. Western blotting

T24 and EJ-1 cells were treated with T4 for 48 h. The cells were collected and the total protein amount was evaluated using BCA reagent (PC0020, Solarbio, China). After protein separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the samples were transferred to PVDF membrane (Millipore, USA), shaken well in TBST containing 5% skim milk, and after 1 h, the following monoclonal antibodies were added: anti-human β-actin (1:5000; Cell Signaling Technology, USA), anti-TP53 (1:1000; Cell Signaling Technology, USA), anti-VEGF (1:1000; Cell Signaling Technology, USA), and anti-αV (1:1000; Cell Signaling Technology, USA). Subsequently, the secondary antibody and horseradish peroxidase were incubated at room temperature. After 2 h, the membrane was washed 5 times with 1× TBST buffer for 5 min each time. Finally, the protein expression was observed using the enhanced chemiluminescence kit (PE0010, Solarbio, China). The band density was quantified using Image J. The grayscale values of the protein bands in the experimental group were compared with those in the PBS group, with all proteins in the PBS group normalized.

2.7. Xenograft BC mouse model

Immunodeficient mice were purchased from SiPeifu (Beijing) Biotechnology Co., Ltd. (China). EJ-1 cells were injected subcutaneously into the right shoulder of each mouse at a dose of 1×10^6 cells per mouse. All mice were randomly divided into two groups ($n = 5$). The experimental group was intraperitoneally injected with 2 mg/mouse of T4 every day, and the control group was intraperitoneally injected with the same amount PBS. During this period, changes in mouse tumor volume were recorded every 3 days, and tail blood was collected and stored every 7 days. After undergoing T4 treatment for 30 days, they were immediately euthanized, and the tumors were collected and photographed and weighted. The formula for calculating tumor volume is as follows:

$$\text{Tumor volume} = \frac{(length) \times (width)2}{2}$$

2.8. Enzyme-linked immunosorbent assay

Blood was taken from the mouse tail and centrifuged for serum separation, and the concentrations of T3, T4, and TSH in the mouse serum were detected using Jianglai Bio-enzyme-linked immunosorbent assay kit.

2.9. Statistical analysis

The statistical analysis for this study was conducted using GraphPad Prism 8. The data were presented as mean ± standard deviation of three independent biological replicates. The Student t-test was used to analyze statistical differences between two groups, while one-way analysis of variance was utilized for comparisons involving three or more groups. All statistical tests were two-sided, and a $p<0.05$ was considered statistically significant.

3. Results

3.1. Tetramethyl thyroxine promoted proliferation of T24 and EJ-1 cells

T24 and EJ-1 cells were treated with varying concentrations of T4 to examine its effects on BC cell proliferation. The results indicated that after 24 h and 48 h of treatment, the proliferation rates of T24 and EJ-1 cells in the experimental

Table 1. Primers used for qRT-PCR

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
GAPDH	AAGGTGAAGGTCGGAGTCAA	GGAAGATGGTGATGGGATTT
TP53	GTTCCGAGAGCTGAATGAGG	TCTGAGTCAGGCCCTTCTGT
VEGF	TCCGAAACCATGAACTTTCTGC	GTAGCTGCGCTGATAGACATCC
αV	AGGCTGATTTCATCGGGGTTGT	AGTTGAGTTCCAGCCTTCATTG

Abbreviation: qRT-PCR: Quantitative real-time polymerase chain reaction.

group were significantly higher than those in the control group, with notable differences observed after 48 h (Figure 1, $p<0.01$). Specifically, the proliferation rates of T24 cells were found to be between 5% and 8.5% at 24 h and between 22.8% and 28.3% at 48 h (Figure 1A), while the proliferation rates of EJ-1 cells ranged from 4.7% to 8.5% at 24 h and from 13.6% to 18.7% at 48 h (Figure 1B). These data indicated that BC cells exhibit accelerated proliferation following T4 treatment, with the effect becoming more pronounced over time. Based on these experimental results, we selected T4 concentrations of 10 nM and 100 nM for subsequent experiments.

3.2. Tetramethyl thyroxine inhibited apoptosis of T24 and EJ-1 cells

To further explore the effect of T4 on apoptosis in BC cells, T24 and EJ-1 cells were treated with 10 nM and 100 nM concentrations of T4 for 48 h. Apoptotic rates were then assessed using flow cytometry. The analysis revealed a marked inhibition of apoptosis in both cell lines following T4 treatment. After 48 h, the apoptosis rates of T24 cells at 10 nM and 100 nM concentrations were 2.79% and 1.66% significantly lower than that of the control group (5.14%; Figure 2A), and the apoptosis rates of EJ-1 cells were 1.81% and 0.58% significantly lower than that of the control group (3.65%; Figure 2B). In summary, the apoptosis of BC cells was inhibited by T4.

3.3. Tetramethyl thyroxine affects migration ability of T24 and EJ-1 cells

We used 10 nM and 100 nM T4 to treat cells to examine its effect on cell migration. Based on the observation from the microscope, the addition of T4 significantly enhanced the migration of T24 and EJ-1 cells. Image J was used to quantitatively analyze the cell scratch healing rate of BC cells after 0 h, 24 h, and 48 h. The scratch healing rates of T24 cells after 24 h were 14.39% and 19.97%, and the scratch healing rates of cells after 48 h were 31.5% and 48.75%, both higher than the 4.66% and 7.74% of the control group (Figure 3A). For EJ-1 cells, the cell scratch healing rates in the control group were only 4.1% and 20.62%. However, after T4 treatment, the cell scratch healing rates at 24 h reached 15.57% and 33.37%, and the cell scratch healing rate at 48 h increased to 33.79% and 56.42% (Figure 3B). The addition of T4 significantly enhanced the migratory capacity of T24 and EJ-1 cells, with this effect becoming more pronounced over time. This suggested that T4 may facilitate tumor progression by promoting the migration of BC cells.

3.4. The expression of TP53, integrin αV and VEGF were significantly elevated in T24 and EJ-1 cells under T4 treatment

Building upon the results of the functional assays, we conducted further investigations to elucidate the molecular mechanisms underlying the effects of T4 on BC cells. Specifically, we examined changes in the expression of key tumor-related genes and proteins in T24 and EJ-1 cells following treatment with 10 nM and 100 nM T4. At the transcriptional level, qRT-PCR was used to assess the mRNA expression of TP53, ITGAV (αV), and VEGFA (VEGF). Compared to the control group, T4 treatment at concentrations of 10 nM and 100 nM significantly downregulated TP53 while upregulating αV and VEGF, with mRNA expression levels showing statistical significance ($p<0.01$; Figures 4A and B). To determine whether these transcriptional changes were reflected at the protein level, we performed western blot analysis followed by quantitative densitometry using Image J software. The results showed a consistent pattern with the mRNA data, in which TP53 protein levels were significantly reduced after T4 treatment, whereas αV and VEGF protein levels were substantially elevated in both T24 and EJ-1 cells. With the increase in T4 concentration, the expression levels of αV and VEGF proteins were further elevated, while the expression level of TP53 protein was further reduced ($p<0.01$; Figure 4C and D). Overall, these findings provide compelling evidence that T4 not only influences the functional behavior of BC cells but also drives specific transcriptional and translational changes that favor tumor progression.

3.5. Tetramethyl thyroxine promotes tumor growth in BC xenograft models

To further demonstrate the tumor-promoting effect of T4 *in vivo*, we utilized EJ-1 cells to establish a BC xenograft

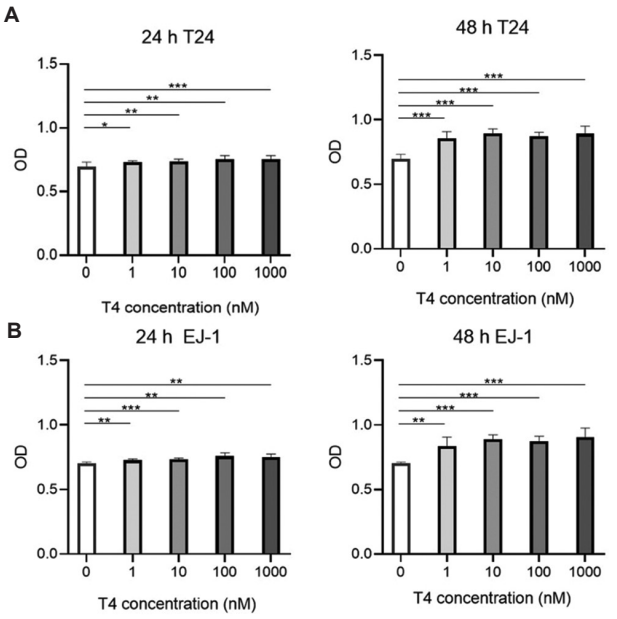

Figure 1. T4 promotes proliferation of T24 and EJ-1 cells. (A) CCK-8 detected that different concentrations of T4 treatment for 24 h and 48 h significantly promoted the proliferation of T24 cells. (B) CCK-8 detected that different concentrations of T4 treatment for 24 h and 48 h significantly promoted the proliferation of EJ-1 cells (OD: 450 nm). Notes: $n = 3$; *$p<0.05$; **$p<0.01$; ***$p<0.001$.
Abbreviation: CCK-8: Cell viability by cell counting kit-8.

tumor model. Tumor-bearing mice were divided into a control group and a T4 treatment group, with five mice in each group. Changes in tumor volume were recorded every 3 days. By the 30th day, the tumor volume in the T4 treatment group reached 545.47 mm³, compared to 251.5 mm³ in the control group (Figure 5A and B). On the 30th day, the mice were sacrificed, and the tumors were excised and weighed. The mass of the tumor in the T4 group post-treatment (0.58044 g) was significantly higher than that in the untreated group (0.29566 g; $p<0.01$; Figure 5A and C). In addition, we collected tail blood samples from the mice to measure the concentrations of T4, T3, and TSH in the serum, with T4 serving as the precursor to T3. The observed increase in serum T4 and T3 levels, along with a decrease in TSH (Figure 5D), indicates signs of hyperthyroidism.[11] The experimental results revealed that by the 28th day, the serum levels of T4 and T3 had significantly increased, while the concentration of TSH had decreased. T4-induced hyperthyroidism in mice may influence the course of tumor development.

4. Discussion

BC is one of the most common malignancies of the urinary system and represents a significant global public health issue. Chronic infections, pathogens, and various diseases

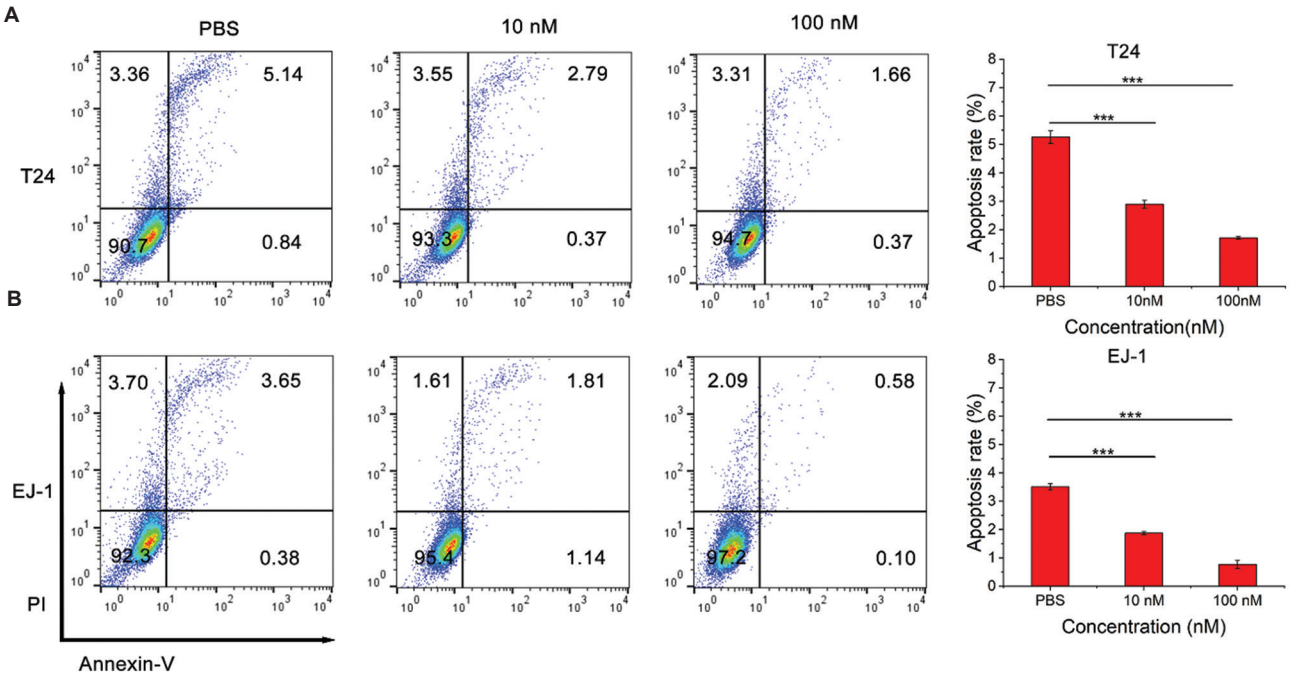

Figure 2. Inhibition of apoptosis in T24 and EJ-1 cells after T4 treatment. (A) The apoptosis rate of T24 cells was significantly inhibited by 10 nM and 100 nM T4 treatment for 48 h. (B) The apoptosis rate of EJ-1 cells was significantly inhibited by 10 nM and 100 nM T4 treatment for 48 h. Notes: $n = 3$; ***$p<0.001$.

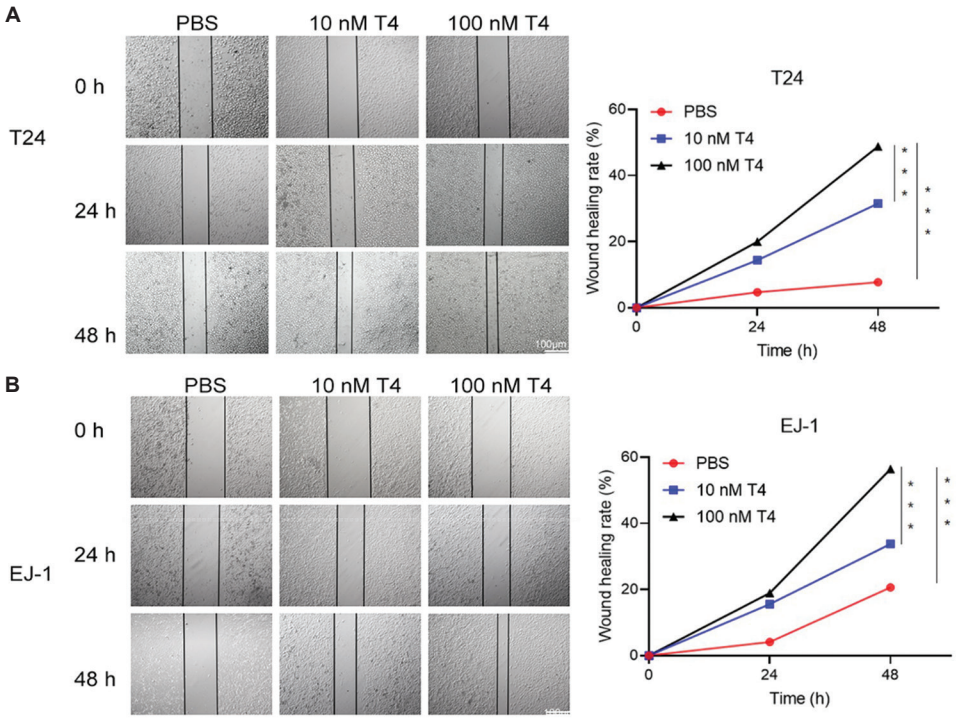

Figure 3. T4 promotes migration of T24 and EJ-1 cells. (A) Treatment of T24 cells with 10 nM and 100 nM for 48 h promoted cell scratch healing. Scale bar: 100 μm. (B) Treatment of TEJ-1 cells with 10 nM and 100 nM for 48 h promoted cell scratch healing. Scale bar: 100 μm. Notes: $n = 3$; ***$p<0.001$.

are prevalent causative factors associated with BC. The pathogenesis of BC is a complex process characterized by mutations and abnormal expression of multiple genes. In the epithelial cells of BC, the high expression of oncogenes such as FGFR3 and PKM2, coupled with the low expression of tumor suppressor genes such as *TP53* and *FBXW7*, constitutes key molecular events in the disease's development. Notably, the mutation rate of the *FGFR3* gene in BC is significantly higher than that of other genes. These genes primarily regulate critical biological processes, including cell proliferation, the cell cycle, apoptosis, and embryonic development.[12,13]

Tumor-associated macrophages and fibroblasts promote tumor development and invasion by secreting cell growth factors such as TGF-β1.[14] In addition, DNA methylation levels and circulating tumor DNA are also related to the occurrence of BC. Research on the mechanisms of BC is expected to provide new strategies and methods for the diagnosis and treatment of BC patients. Yu *et al*. exposed human normal urothelial cells to cigarette smoke and found that their morphology changed. Cigarette induction enhanced their migration and invasion capabilities while reducing the expression of epithelial markers and increasing the expression of mesenchymal markers.[15] Liu *et al*. revealed that the transcription factor MYBL2 promotes the proliferation and metastasis of BC by activating the downstream target gene *CDCA3*.[16] Yang *et al*.'s study demonstrated that mesenchymal stem cells in the tumor microenvironment promote BC cell progression by enhancing mitochondrial function.[17] T4 is associated with the prevalence of various tumors, including THCA, breast cancer, and liver cancer.[18] We aimed to explore the relationship between T4 and BC and its mechanism of action.

T4 is a hormone primarily secreted by the thyroid gland, playing a crucial role in the body's metabolism,[19] growth, development,[20] and nervous system function.[21] In the bloodstream, approximately 99.7% of T4 is bound to plasma proteins, serving as the storage and transportation form of the hormone, which possesses no biological activity. A small fraction of free T4 in the blood is capable of passing through cell membranes and entering tissue cells to exert its biological effects.[22]

Currently, the relationship between T4 and cancer is an active area of interest in the field of medical research. According to existing research, T4 is associated with angiogenesis and cell proliferation, and it regulates both the physiological processes of normal cells and the proliferation of tumor cells.[18] T4 regulates cancer cell proliferation through genomic and non-genomic pathways. The genomic pathway involves the intracellular conversion of T4 to T3 through deiodination, followed by

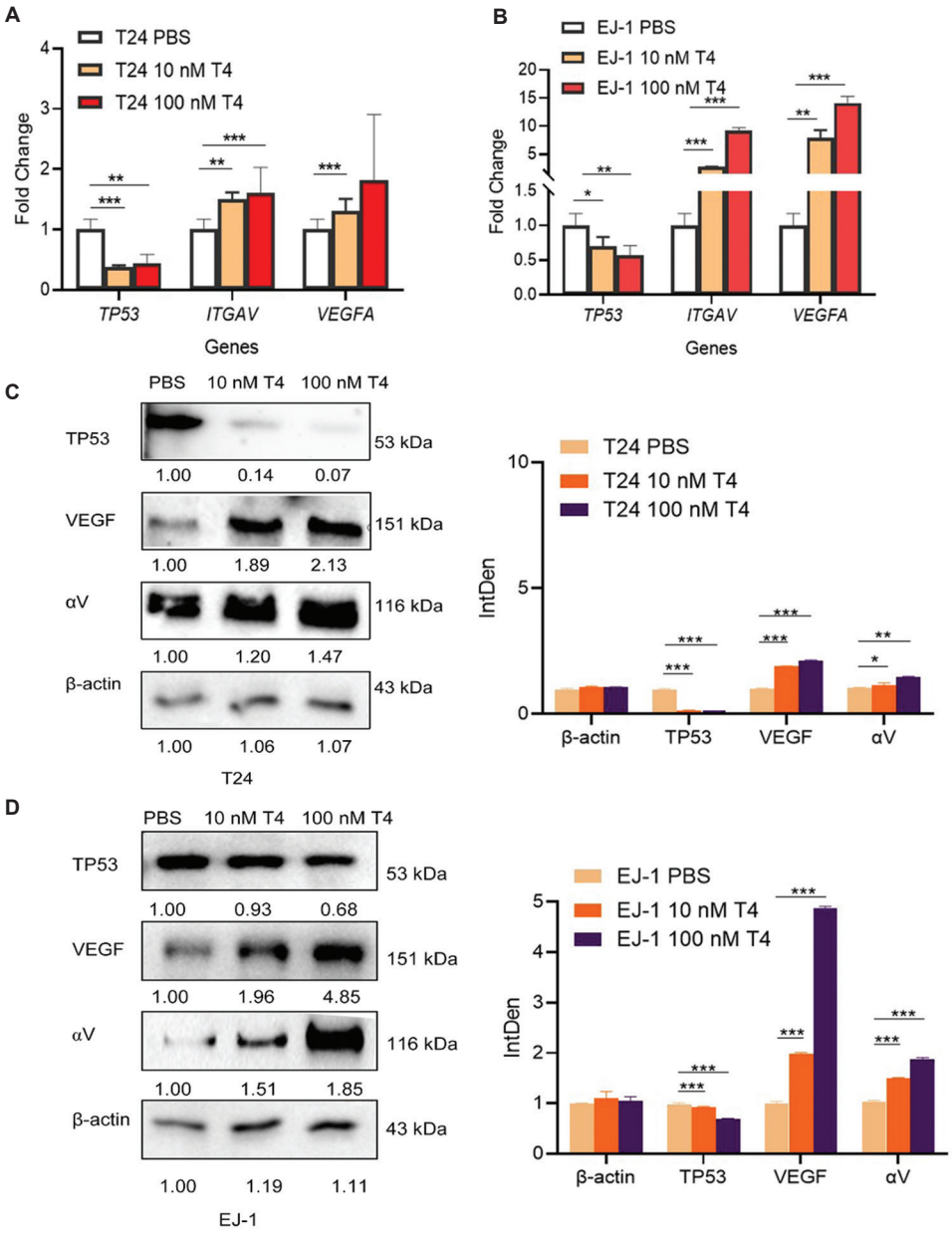

Figure 4. T4 inhibits apoptosis by upregulating the expression of VEGF, αV and downregulating TP53 in T24 and EJ-1 cells. (A) Increased ITGAV (*αV*) and *VEGFA* (*VEGF*) mRNA levels and decreased *TP53* mRNA levels in T24 cells after T4 treatment detected by qRT-PCR. (B) Increased *ITGAV* (*αV*) and *VEGFA* (*VEGF*) mRNA levels and decreased *TP53* mRNA levels in EJ-1 cells after T4 treatment detected by qRT-PCR. (C) Western blotting analysis of elevated αV and VEGF protein expression and decreased TP53 expression in T24 cells after T4 treatment and related quantitative analysis. (D) Western blotting analysis of elevated αV and VEGF protein expression and decreased TP53 expression in EJ-1 cells after T4 treatment and related quantitative analysis. Notes: $n = 3$; *$p<0.05$; **$p<0.01$; ***$p<0.001$.
Abbreviation: qRT-PCR: Quantitative real-time polymerase chain reaction.

T3 entering the nucleus and binding to receptors Tα and Tβ, thereby regulating the transcription of target genes and promoting cell proliferation and differentiation. The non-genomic pathway refers to the direct action of T4 without conversion to T3, where T4 binds to integrin receptors on the cell membrane, activating downstream signaling pathways (MAPK/ERK and PI3K/AKT), promoting VEGF expression, and enhancing angiogenesis. Ultimately, these pathways enhance the proliferation, migration, and invasion capabilities of tumor cells.[23] Studies by Tang *et al.* have shown that T4 causes phosphorylation by MAPK of nuclear ERα at serine-118 in MCF-7 cells and

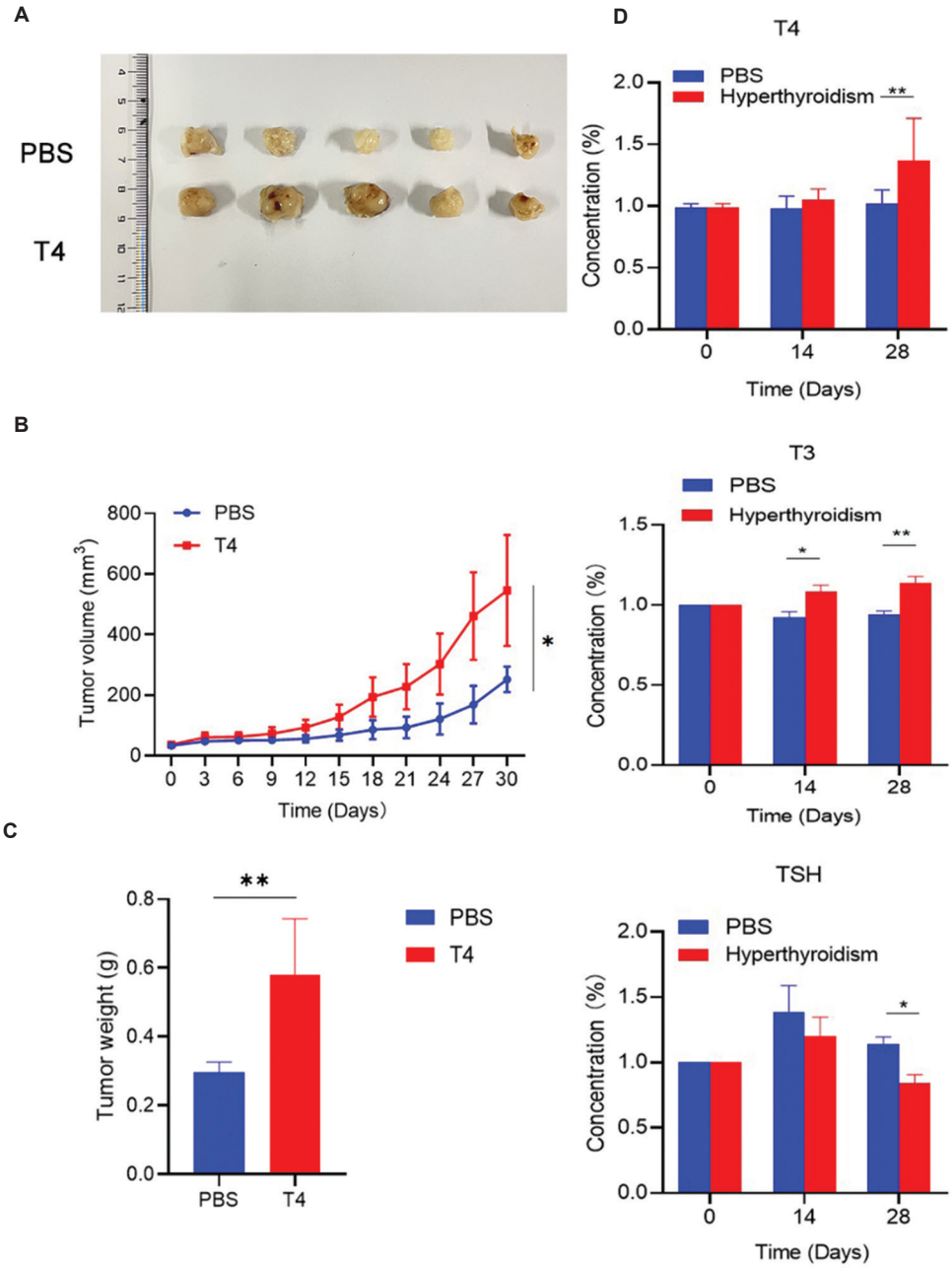

Figure 5. T4 promotes tumor growth in bladder cancer xenograft models. (A) Schematic diagram of tumor resection after T4 treatment. (B) T4 promotes tumor volume growth in xenograft tumor models. (C) Increase in tumor weight after 30 days of T4 treatment. (D) The T4 treatment process detected an increase in T4 and T3 levels and a decrease in TSH levels in the serum of mice. Notes: $n = 5$; *$p<0.05$; **$p<0.01$.
Abbreviation: TSH: Thyroid-stimulating hormone.

promotes cell proliferation through the ER by a MAPK-dependent pathway.[24] In THCA, the interaction of T4 with integrins leads to the inhibition of TP53-dependent apoptosis in tumor cells.[25] Meng *et al.* found that T4 increased proliferating cell nuclear antigen-induced ERα phosphorylation and promoted the proliferation of NCI-H522 and NCI-H510A lung cancer cells.[26] Research by Wang *et al.* demonstrated that TRα transcript levels were significantly increased in primary liver cancer. Further research revealed that T4 activated the NF-κB signaling pathway to initiate the expression of the *BMI1* gene and promote the self-renewal of liver cancer stem cells.[10]

We initially observed through CCK-8 experiments that the proliferation of BC T24 and EJ-1 cells increased

by 5 – 28.3% and 4.7 – 18.7%, respectively, following T4 treatment. Concurrently, the cell migration rates after the co-incubation of T4 with T24 and EJ-1 cells also exhibited increases of 9.27 – 41.01% and 11.47 – 35.8%, respectively. In addition, the apoptosis rates of T24 and EJ-1 cells were significantly reduced after T4 treatment. *In vivo* xenograft tumor models further substantiated that T4 promotes BC tumor formation in BABLC/C nude mice.

Mechanistically, we found that the mRNA expression of integrin αV and VEGF increased, the expression of TP53 mRNA decreased, and the protein levels also showed consistent changes in BC cells after T4 treatment. Integrin αV is a cell surface receptor that belongs to the integrin family. It can form heterodimers with β1, β3, β5, β6, and β8, resulting in the formation of Alpha-V class integrins, which play a significant role in the occurrence and development of tumors.[27] The involvement of integrin αV in tumor development is multifaceted. It can activate TGF-β, a cytokine that is crucial for tumor-associated blood vessel formation. Furthermore, the activation of integrin αV can inhibit apoptosis by modulating interactions with the extracellular matrix.[28] Existing studies have demonstrated that integrin αV is highly expressed in tissues from breast cancer,[29] lung cancer,[28] gastric cancer,[30] colorectal cancer,[31] and esophageal adenocarcinoma,[32] and it promotes the proliferation, invasion, and migration of tumor cells. van der Horst *et al*. found that the knockdown of integrin αV expression in BC cells *in vitro* significantly reduced cell migration ability. The expression of the epithelial marker E-cadherin associated with EMT increased, while the mesenchymal markers N-cadherin and vimentin were downregulated. In addition, the study revealed that the subpopulation of BC cells with high expression of integrin αV typically exhibited higher ALDH activity, with ALDH being a biomarker commonly used to identify cancer stem cells. When integrin αV was knocked down through siRNA, the proportion of ALDH-positive cells significantly decreased, and the expression levels of stem cell-related transcription factors such as NANOG and BMI1 were also markedly downregulated. Furthermore, in the colony formation assay, knockdown of integrin αV significantly inhibited the self-renewal capacity of cells. These results indicated that integrin αV not only promotes the metastasis and invasion behaviors of BC cells by regulating the EMT process but may also play a regulatory role in maintaining the characteristics of cancer stem cells.[33] Sachs *et al*. conducted a clinical evaluation and found that the expression of integrin αV was significantly elevated in high-grade BC and muscle-invasive carcinoma. Particularly, in aggressive tumors, the expression level of integrin αV was positively correlated with the degree of tumor differentiation, proliferative activity, and local invasive capability. Furthermore, follow-up data from some patients showed that those with high integrin αV expression had higher recurrence rates and shorter survival times, suggesting its potential clinical value in prognosis assessment.[34] Integrin αV is highly expressed during angiogenesis and promotes endothelial cell growth and survival.[35] In melanoma cells, integrin αV controls cell survival by inactivating p53 and activating the MEK1 signaling pathway.[36] VEGF is a crucial regulator of tumor angiogenesis, facilitating the development of vascular networks that supply essential oxygen and nutrients to tumor cells. In addition, VEGF can enhance the distant metastasis of tumors by influencing the invasiveness and migratory capabilities of tumor cells, thereby promoting their entry into the bloodstream and lymphatic system.[37] VEGF is a downstream molecule of the MAPK pathway, and the activation of the PI3K/AKT pathway can promote the expression of VEGF.[38] This suggests that T4 may promote the development of BC by activating the MAPK/ERK or PI3K/AKT signaling pathways through binding to integrin αV. This points the way for further mechanistic research. As a tumor suppressor, TP53 prevents the occurrence and progression of tumors by inducing G1 phase arrest in the tumor cell cycle and inhibiting the proliferation of damaged tumor cells.[39] In summary, T4 promotes the angiogenesis of BC cells by activating integrin αV. The high expression of VEGF, an angiogenic factor, supports this assertion. Concurrently, the increased expression of VEGF enhances the migratory capacity of BC cells. In addition, the activation of integrin αV and the inhibition of TP53 contribute to the suppression of apoptosis.

Thyroid hormones can serve as an energy source for intestinal cells by binding to short-chain fatty acids, which are metabolites of anaerobic microbial fermentation, thereby inducing intestinal cell differentiation.[40] In addition, thyroid hormones can regulate the Wnt/β-catenin signaling pathway through TRα1, affecting the self-renewal of intestinal stem cells.[41] Thyroid hormones also modulate the proliferation and activation of T lymphocytes by influencing the NF-κB and protein kinase C signaling pathways, as well as β-adrenergic receptors.[42] It is evident that thyroid hormones exert specific effects on the proliferation of normal cells in different tissues. By increasing normal urothelial cells for comparative studies, further investigation into the differences in the mechanisms and effect intensities of T4 in normal urothelial cells and BC cells can clarify the tissue specificity of T4. In future studies, we could incorporate multiple BC cell lines (such as J82, 5637, and HT1376) and primary BC cells to conduct *in vivo* and *in vitro* experiments, thereby enhancing the generalizability of the research findings and elucidating the promoting

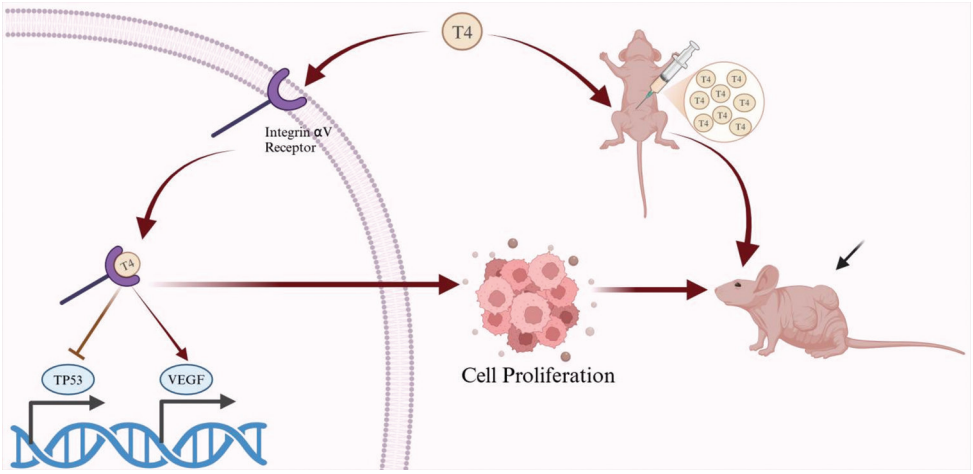

Figure 6. The molecular mechanism of T4 promoting the occurrence of bladder cancer

effects of T4 on different types of BC, with particular attention to the responses of patient-derived xenografts to T4. The BBN-induced orthotopic BC model, which simulates the prolonged process of tumorigenesis, serves as an ideal platform for assessing the role of carcinogenic factors. In this mouse model, intraperitoneal injection of T4 intervention allows direct observation of whether thyroid hormones influence the initiation and progression of BC.[43] Simultaneously, the expression of BC markers such as ERCC1 and TP53 after T4 treatment is detected, or in animal models, the correlation between marker levels and tumor growth rate, recurrence rate, and other indicators is analyzed to provide an experimental basis for prognosis assessment. Mutations in the *TP53* gene and aberrant activation of the FGFR3 signaling pathway are frequently observed in BC cells. These molecular alterations are closely associated with changes in cell proliferation, apoptosis, and invasion. Further research into the roles of TP53 and FGFR3 in these processes will help to elucidate their contribution to BC progression. Such studies will provide a theoretical foundation for the development of novel therapeutic targets and treatment strategies.[44] Second, retrospective or prospective clinical studies are carried out to compare thyroid function parameters (especially serum T4 and TSH levels) between BC patients and age-matched healthy individuals. Analyze whether the proportion of subclinical hyperthyroidism (low TSH, high normal T4) is higher among BC patients, or whether there is a correlation between T4 levels and BC staging/prognosis. If it is found that elevated thyroid hormone levels or thyroid dysfunction in BC patients are associated with tumor severity, it would suggest that T4 may have clinical relevance in tumor development and progression. Through the above research directions, we hope to effectively address the limitations of current research in future studies, further elucidate the biological mechanisms by which T4 hormone regulates BC through the αV and VEGF pathways, and provide important reference for future clinical translation.

This study confirmed through *in vivo* and *in vitro* experiments that T4 enhances the proliferation, migration, and tumorigenic ability of BC cells by upregulating the expression of integrin αV and VEGF and downregulating TP53 expression. However, it is noteworthy that the role of thyroid hormones in tumor biology remains complex and bidirectional. A recent study demonstrated that T3 exhibits significant anti-tumor effects in a hepatocellular carcinoma model. The study found that T3 can activate the LKB1/AMPK/Raptor pathway by inducing thyroid hormone response stimulatory protein, thereby inhibiting the abnormal activation of the PI3K/Akt/mTOR pathway, ultimately blocking the ENO_2-induced aerobic glycolysis process, and thus slowing down the metabolic progression and growth of tumors.[45] This study suggested that thyroid hormones may exhibit opposing biological effects depending on different tissue types, receptors, or signaling pathway dependencies. This discrepancy may be partially attributed to the distinct receptor selectivity and signaling mechanisms of T3 and T4. T3 primarily mediates gene transcription regulation by binding to nuclear thyroid hormone receptors (TRα/TRβ), whereas T4 has been found to bind to the integrin αVβ3 receptor in a non-genomic manner, rapidly activating signaling pathways such as MAPK/ERK and PI3K/AKT, thereby promoting cell proliferation and angiogenesis.[46,47] Therefore, even as thyroid hormones, their impact on tumors may exhibit tissue specificity, dose dependency, and receptor distribution dependency. In this study, sustained T4 treatment in a mouse model led to elevated

serum T4 and T3 levels and decreased TSH levels, exhibiting a hyperthyroidism-like hormonal state, which may affect multiple organ systems and indirectly participate in regulating the tumor microenvironment. For example, by altering immune cell activity, vascular permeability, oxidative stress status, etc., all of which may promote the tumor development process. A systematic review and meta-analysis indicated an increased risk of breast cancer in hyperthyroidism patients.[48] In these patients, THCA exhibited more aggressive behavior, with higher rates of local invasion and poor prognosis.[49]

In summary, our study has revealed the tumor-promoting role of T4 in BC. However, we also acknowledge that T4 does not exhibit a tumor-promoting effect across all tumor types, especially when compared with T3, as there are fundamental differences in their mechanisms of action and receptor dependency. Future research should further explore the functional similarities and differences between T4 and T3 in various cancer types; clarify their receptor-mediated mechanisms; and investigate the correlation between thyroid function status and the occurrence, progression, and prognosis of BC in clinical cohorts, to provide explanations with greater physiological relevance and translational value.

5. Conclusion

In summary, our initial *in vitro* experiments revealed that T4 can promote the proliferation and migration of BC cells while inhibiting their apoptosis. Subsequent *in vivo* experiments confirmed that increased serum concentrations of T4 in mice also promote the formation of BC tumor. Further mechanistic analysis indicated that T4 facilitates the progression of BC by binding to the integrin receptor αV, which in turn influences the expression of VEGF and TP53 (Figure 6).

For the 1st time, we have demonstrated through both *in vivo* and *in vitro* experiments that T4 can enhance the development of BC and elucidate its molecular mechanism, thereby laying the groundwork for improved prevention, diagnosis, treatment, and prognosis assessment of this disease.

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Conflict of interest

The authors declare that no commercial or financial conflicts of interest were identified in this research.

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Formal analysis: Wenjing Zhang
Investigation: Wenjing Zhang
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Validation: Wenjing Zhang
Visualization: Wenjing Zhang
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Writing–editing & review: All authors

Ethics approval and consent to participate

The ethical approval has been obtained from the ethical committee member board of the Animal Care Review Committee of the China-Japan Friendship Hospital and conducted in accordance with its recommendations and ethical regulations. All experimental protocols were approved by the ethical committee member board of the Animal Care Review Committee of the China-Japan Friendship Hospital. The mice were maintained under standard conditions according to the institutional guidelines for animal care (Ethics code: 2021-42-K26).

Consent for publication

Not applicable.

Availability of data

The data that support the findings of the study are available from the corresponding author by e-mail upon reasonable request.

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