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T-box transcription factor 2 mediates antitumor immune response in cutaneous squamous cell carcinoma by regulating the expression of programmed death ligand 1

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Abstract

Background: Cutaneous squamous cell carcinoma (CSCC) is the second largest nonmelanoma skin cancer in humans; effective treatment options for metastatic CSCC are still in short. In this study, we aimed to explore the function of T-box transcription factor 2 (TBX2) in CSCC.

Methods: The expression level of TBX2 was determined in CSCC samples and cell lines. Programmed death ligand 1 (PD-L1) expression was also analyzed in human CSCC samples. Furthermore, SCC13 cells were transfected with TBX2-DN (loss of function) or normal TBX2 to check its role in regulating PD-L1.

Results: The expression level of TBX2 was positively correlated with the stage of CSCC. CSCC tumor cell lines have significantly higher expression levels of TBX2 than normal skin cell lines, and SCC13 cells showed the highest expression. PD-L1 expressions were upregulated during the progression of CSCC, and positively correlated with TBX2. Furthermore, PD-L1 expression increased in SCC13 cells overexpressing TBX2. However, TBX2 did not regulate the activation of IFNγ signal, but mediated the expression of interferon regulatory factor 1 (IRF1) and PD-L1 in both SCC13 and PDV cells.

Conclusion: TBX2 could mediate antitumor immune response in CSCC by regulating the expression of PD-L1 through IRF1. It might be a prognostic marker in CSCC and synergistic target for PD-1 immunotherapy.

KEYWORDS
cutaneous squamous cell carcinoma, immunotherapy, PD-L1, TBX2

1 | INTRODUCTION

Squamous cell carcinoma is a malignant tumor that occurs in epidermis or adnexal cells. It can occur in the skin, lung, esophagus, prostate, and other epithelium tissues. Cutaneous squamous cell carcinoma (CSCC) is the second largest nonmelanoma skin cancer in humans.[1] Cumulative Ultraviolet Rays (UV) exposure (mainly from sunlight) and immunosuppressive status are the main risk factors for CSCC. Most CSCC can be surgically removed and treated. Even if CSCC has local lymph node metastasis, it can still be treated with surgery. But if it has distant metastasis that cannot be operated surgically, it needs to be treated with chemotherapy, radiotherapy, or immunotherapy, etc. At present, effective treatment options for metastatic CSCC are still in need.

Immunotherapy has been a promising strategy for many cancers including CSCC.[2] PD-1 inhibitors have been used in the treatment

of CSCC, but the complete response rate is only about 10%. The antiprogrammed death ligand 1 (PD-1) drug Keytruda (pembrolizumab) has been approved by the US Food and Drug Administration (FDA) for CSCC treatment.[3] Another anti-PD-1 antibody drug Libtayo (Cemiplimab-rwlc) has shown good efficacy in the treatment of skin squamous cell carcinoma[4]; thus the FDA has approved Libtayo for metastatic CSCC that is not suitable for surgery or radiotherapy.[5] The results of its phase II clinical trial showed that 34% of patients receiving immunotherapy have been significantly improved. In a word, immunotherapy has great potential in the treatment of CSCC. However, there are many factors that affect the patient response to immunotherapy, it is urgent to understand the mechanism and develop new therapeutic strategy thus improve the efficacy of immunotherapy for CSCC.

Comparing the gene expression profile of CSCC samples with normal skin tissues, we found high expression of T-box transcription factor 2 (TBX2) correlated with clinical stage of CSCC. TBX2 has been reported to be upregulated in a variety of cancers.[6] It is a T-box family member, a cell death repressor that functions in bypassing cell growth control. In neuroblastoma, TBX2 is a core regulatory, downregulation of TBX2 can lead to massive cell apoptosis.[7] In gastric cancer, TBX2 expression can predict tumor recurrence and adjuvant chemotherapy benefits.[8] While in melanoma, TBX2 has high expression in tumor cells and plays a key role in promoting proliferation of tumor cells while suppressing cell senescence.[9] Moreover, TBX2 confers poor prognosis and promotes temozolomide resistance in glioblastoma.[10] But its role in CSCC has not been reported. Recently, *Tbx2* is found to be associated with cancer immuno phenotypes.[11] Thus, in this study, we sought to explore the role of TBX2 in immunotherapy.

2 | MATERIALS AND METHODS

2.1 | Patient sample collection

Tumor biopsy samples from CSCC patients in stage I, II, III/IV (10 samples of CSCC-I, 21 samples of CSCC-II, 18 samples of CSCC-III/IV) and six normal human skin tissue samples were collected, then stored at −80°C for later analysis. Characteristics of normal controls and CSCC patients are described in Table 1. All procedures were approved by the Ethics Committee of the Affiliated Qingdao Municipal Hospital of Qingdao University. Written informed consent was obtained from each patient or volunteer.

2.2 | Cell lines

HEKa, HEKn, and A431cell lines were purchased from ATCC (Manassas, VA) and cultured following protocols from ATCC. COLO16 cell line was purchased from Tongpai Biotechnology (Shanghai, China) following the previous instruction.[12] Cells were cultured with RPMI medium 1640 with 5% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) and 1% Penicillin–Streptomycin–Glutamine. DJM-1 cell line was purchased from Shanghai Yunhao Biotech (Shanghai, China). Cells were cultured in DMEM supplemented with 10% FBS. SCC13 was obtained from Chinese Academy of Medical Sciences and cultured as previous described.[13] All cells were authenticated by short tandem repeat profiling and were used within 6 months of continuous passage. For proliferation assay, cells were seeded into 96-well plates at 1500 cells each well. After 48 h of growth, the media was replaced with 100-μl media containing 10-μl CCK-8 reagent (ab228554, Abcam, Cambridge, MA) and incubated for 2 h, then analyzed at 450 nm.

TABLE 1 Characteristics of normal controls and cutaneous squamous cell carcinoma (CSCC) patients

Characteristics	Number	%
Normal controls	6	
Age (years)		
<65	2	33.3
≥65	4	66.7
Sex		
Male	5	83.3
Female	1	16.7
Location of biopsy samples		
Forehead	2	33.3
Neck	3	50.0
Cheek	1	16.7
CSCC patients	49	
Age (years)		
<65	8	16.3
≥65	41	83.7
Sex		
Male	43	87.8
Female	6	12.2
Location of biopsy samples		
Forehead	7	14.3
Neck	14	28.6
Cheek	12	24.5
External auditory canal	3	6.1
Scalp	6	12.2
Nose	5	10.2
Back	2	4.1
Clinical stage		
I	10	20.4
II	21	42.9
III/IV	18	36.7

2.3 | Cell transfection

The wild-type human TBX2 and dominant negative isoform TBX2-DN lacking aa 1–301 were cloned into pcDNA3. The plasmids were then transfected into human SCC13 cells. The cells were harvested for analysis 48 h after transfection.

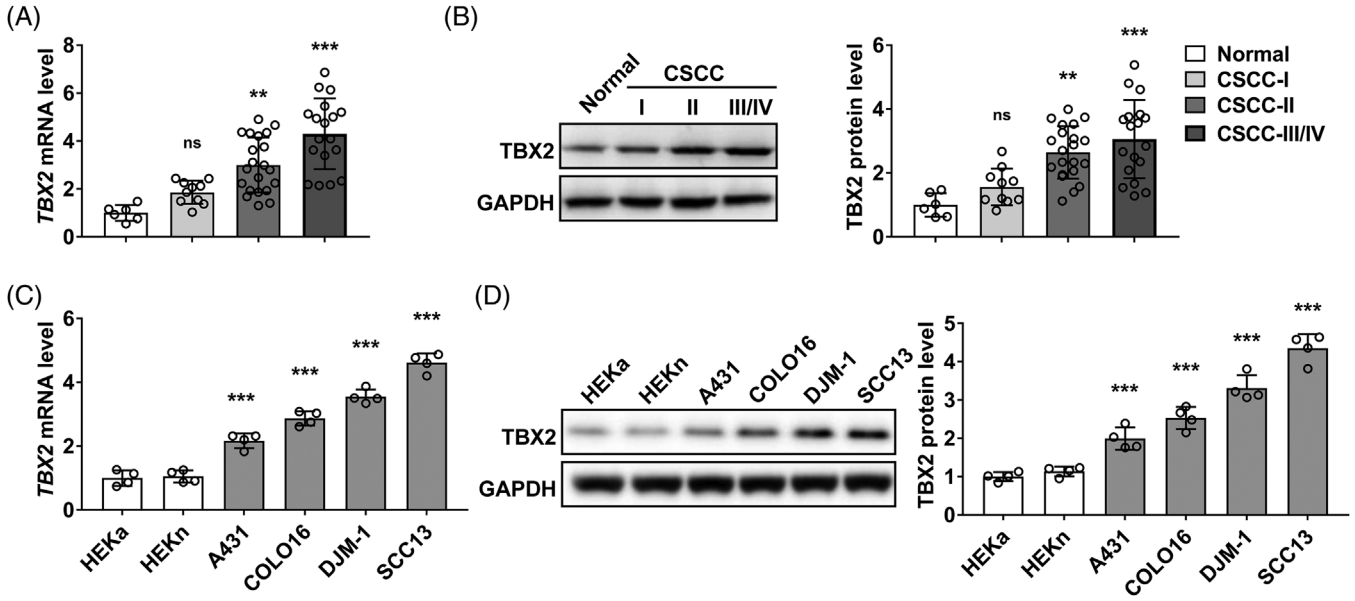

FIGURE 1 TBX2 was upregulated in cutaneous squamous cell carcinoma (CSCC) specimens. (A and B) Relative TBX2 mRNA (A) and protein (B) level in normal or CSCC specimens in different stages were determined by RT-qPCR and western blot, respectively. Six normal skin specimens, 10 of CSCC-I, 21 of CSCC-II, 18 of CSCC-III/IV. (C and D) Relative TBX2 mRNA (C) and protein (D) level in cultured HEKa, HEKn, A431, COLO16, DJM-1, and SCC13 cells were determined by RT-qPCR and western blot, respectively. $n = 4$. ns/*, the significance compared to normal specimens or HEKa cell was analyzed by one-way ANOVA test.

The dominant negative mouse Tbx2 (Tbx2-DN) was cloned into retroviral vector pBABE-puro and then packaged into retroviruses. PDV cells were infected with retroviral Tbx2-DN or control viruses, followed by puromycin treatment at 2 μg/ml for 3 days. The stable cell line was established after two passages.

The knockdown of interferon regulatory factor 1 (IRF1) in SCC13 cells was achieved by infection of lentiviruses carrying shRNAs targeting human IRF. Human IRF shRNA (TRCN0000014672) and control viruses (SHC016V) were purchased from Sigma (St. Louis, MO).

2.4 | mRNA expression detection

RNA samples were extracted using an RNA extraction kit (#74004, Qiagen, Valencia, CA), and RNA concentrations were determined using UV absorbance measurements at 260 nm. Then, the reverse transcription kit (#639506, Takara, Dalian, China) was used to reverse transcribed mRNA into cDNA, and the qPCR reaction kit QUICK GreenER Two-Step Real Time Quantitative PCR (qRT-PCR) Kit ARD (#11763, Invitrogen, Waltham, MA) was used to detect the mRNA expression levels of indicated genes.

2.5 | Cell line-derived xenograft mouse model

PDV cells were transduced with empty vectors (Vec) or Tbx2-DN-encoding lentivirus. Cells with stable expression of vectors were selected and 1×10^7 PDV cells for each mouse were injected subcutaneously following a previous protocol.[14] Tumor volume and tumor weight were checked at day 49 when tumors were harvested for analyzing. Animal studies were approved by the Ethics Committee of the Affiliated Qingdao Municipal Hospital of Qingdao University.

2.6 | Protein expression analysis

Protein samples from patient tumors or cell lines were extracted using RIPA lysis buffer containing protease inhibitors. Then the western blotting experiment was performed according to the standard protocol. Antibodies include anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (#2118, Cell Signaling, Danvers, MA), anti-TBX2 (#sc-514291, Santa Cruz, Dallas, TX), antihuman PD-L1 (#13684, Cell Signaling), antimouse PD-L1 (#60475, Cell Signaling), anti-IRF1 (#8478, Cell Signaling), anti-STAT1 (#14994, Cell Signaling), and anti-pSTAT1 (#7649, Cell Signaling). GAPDH was used as a loading control.

2.7 | Flow cytometric analysis

Cell-derived xenograft tumors were collected at 49d post-cell transplantation. Samples were dissociated with Tumor Dissociation Kit (#130-096-730, Miltenyibiotec, Bergisch Gladbach, Germany), single cell solution was prepared for FACS analysis as previous described.[15] Briefly, Fc receptors were blocked by antimouse CD16/CD32 antibodies (#553142, BD Biosciences, Franklin Lakes, NJ), then washed

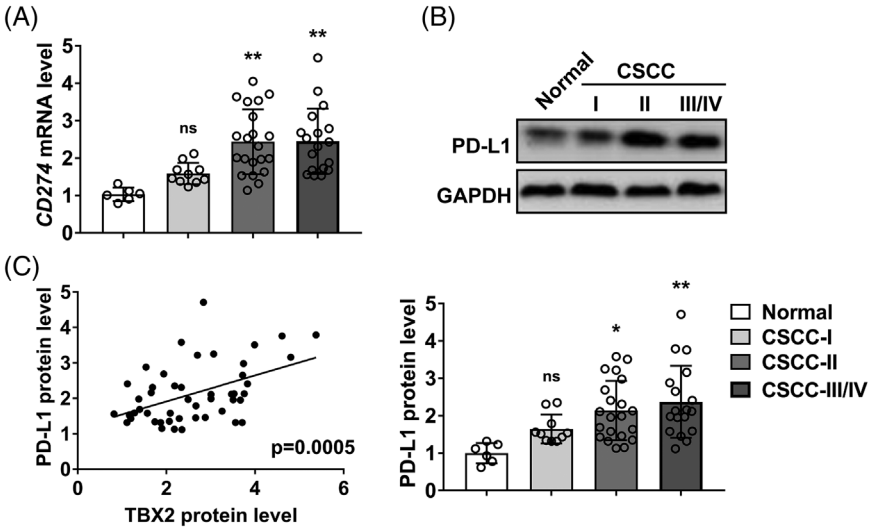

FIGURE 2 TBX2 level was correlated with programmed death ligand 1 (PD-L1) in cutaneous squamous cell carcinoma (CSCC) specimens. (A and B) Relative PD-L1 (CD274) mRNA (A) and protein (B) level in normal or CSCC specimens in different stages were determined by RT-qPCR and western blot, respectively. Six normal skin specimens, 10 of CSCC-I, 21 of CSCC-II, 18 of CSCC-III/IV. ns/*, the significance compared to normal specimens was analyzed by one-way ANOVA test. (C) Pearson correlation assay of TBX2 and PD-L1 protein level determined by western blot in CSCC specimens

with staining buffer (PBS plus 2% FBS), incubated for 30 min at 4°C with antibodies including PE-CD8 (#100707, Biolegend, San Diego, CA), APC-CD3 (#100235, Biolegend), FITC-PD-1 (#135213, Biolegend). After incubation, samples were washed and resuspended in staining buffer. Flow cytometry analysis was done using a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ), then data were analyzed with FlowJo software.

2.8 | Statistical analysis

Pearson correlation test, two-tailed t-test, two-way Analysis of Variance (ANOVA) test, or one-way ANOVA in GraphPad prism 7 was used for statistical analysis. Data were represented as mean ± standard deviation.

3 | RESULTS

3.1 | TBX2 expression in CSCC specimens

First, we collected tumor tissue samples from CSCC patients at different stages, and normal skin samples from healthy volunteers. Then, we analyzed the mRNA and protein expression levels of TBX2 (Figure 1A,B). It was found that the expression level of TBX2 was positively correlated with the stage of CSCC (relative mRNA level: Stage I: 1.86 ± 0.48, Stage II: 3.00 ± 1.15, Stage III/IV: 4.30 ± 1.48; relative protein level: Stage I: 1.56 ± 0.57, Stage II: 2.65 ± 0.82, Stage III/IV: 3.06 ± 1.22). As the disease progressed, the expression level increased. In addition, compared with normal skin cell lines HEKa and HEKn, CSCC tumor cell lines A431, COLO16, DJM-1 and SCC13 have significantly higher mRNA expression levels and protein expression levels of TBX2 (Figure 1C,D). As SCC13 cells showed the highest expression level of TBX2 compared to HEKa cell (relative mRNA level: 4.61 ± 0.30; relative protein level: 4.35 ± 0.37); therefore, this cell line was used to carry out the following research for the analysis of function and mechanism of TBX2 in CSCC.

3.2 | TBX2 level was correlated with PD-L1 in CSCC specimens

To explore the mechanism by which TBX2 regulates the progress of CSCC, we analyzed the expression level of PD-L1 (gene CD274). The results showed that *CD274* relative mRNA (Figure 2A) (Stage I: 1.59 ± 0.28, Stage II: 2.44 ± 0.86, Stage III/IV: 2.45 ± 0.87) and protein expression levels (Figure 2B) (Stage I: 1.65 ± 0.39, Stage II: 2.14 ± 0.79, Stage III/IV: 2.37 ± 0.96) were upregulated during the progression of CSCC compared to normal skin. And, the protein expression level of TBX2 in tumor tissues was positively correlated with the expression level of PD-L1 protein (Figure 2C) ($p = 0.0005$), suggesting that TBX2 may be involved in regulating the expression of PD-L1.

3.3 | TBX2 mediated the expression of PD-L1 in vitro

In order to further explore the mechanism by which TBX2 regulates the expression level of PD-L1, we overexpressed the loss-of-function form TBX2-DN or wild type (WT) TBX2 proteins, which has the normal function in SCC13 cells, with empty vector transfected cells as control. We then analyzed the expression level of PD-L1 in these three groups. The results showed that the expression level of PD-L1 gene *CD274* relative mRNA in cells overexpressing TBX2-DN was significantly reduced (0.27 ± 0.07), while the expression level in SCC13 cells overexpressing TBX2 was significantly increased (3.55 ± 0.28) (Figure 3A), and the level of PD-L1 protein also showed the same trend (Figure 3B). It indicated that TBX2 may participate in the regulation of PD-L1 expression in CSCC cells.

3.4 | TBX2 mediated the expression of PD-L1 by targeting IRF1

In human CSCC cells, IRF1 has been reported to be a key transcription factor regulating the expression of PD-L1. We tested the

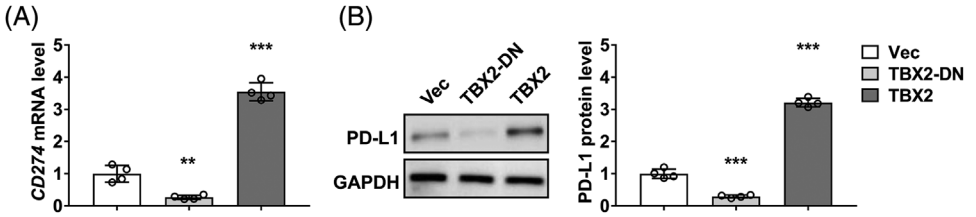

FIGURE 3 TBX2 mediated the expression of programmed death ligand 1 (PD-L1) in vitro. SCC13 cells were transfected with empty vectors (Vec), TBX2-DN (dominant negative)-encoding plasmids or TBX2-encoding plasmids for 48 h, and harvested for analyzing the relative mRNA (A) and protein (B) level of PD-L1 (CD274) by RT-qPCR or western blot, respectively. $n = 4$. *, the significance compared to control was analyzed by one-way ANOVA test.

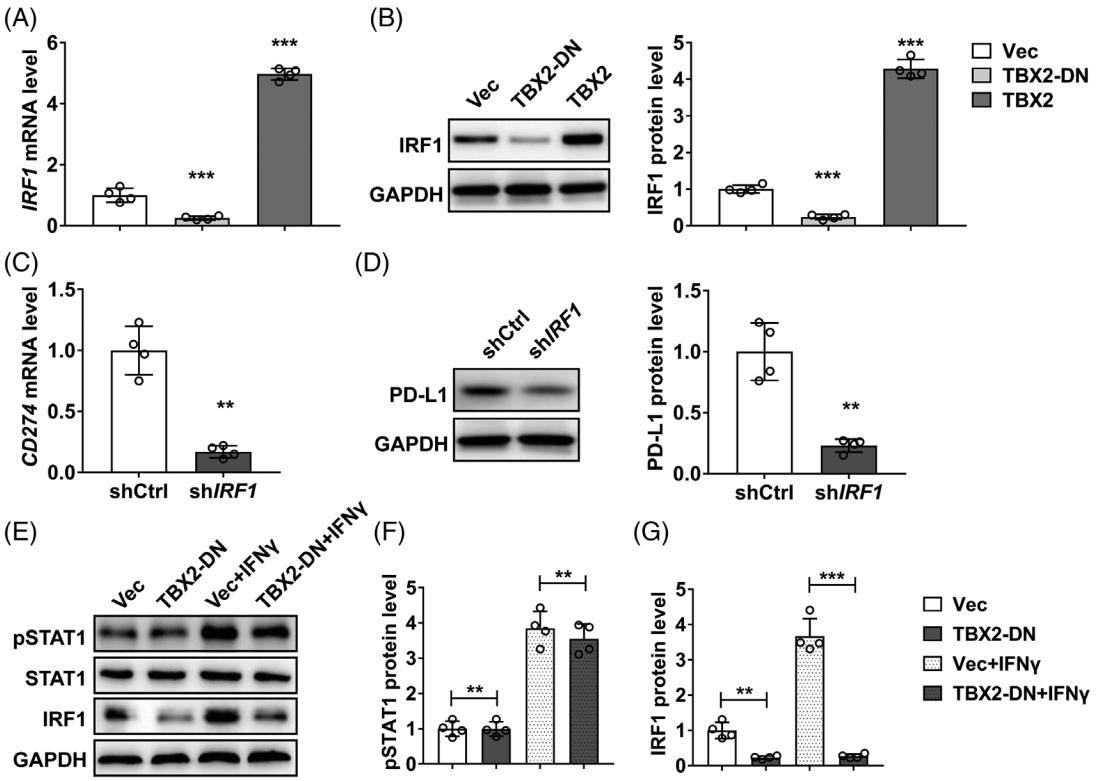

FIGURE 4 TBX2 mediated the expression of programmed death ligand 1 (PD-L1) by targeting IRF1 in human cutaneous squamous cell carcinoma (CSCC) cells. (A and B) SCC13 cells were transfected with empty vectors (Vec), TBX2-DN (dominant negative)-encoding plasmids or TBX2-encoding plasmids for 48 h, and harvested for analyzing the relative mRNA (A) and protein (B) level of IRF1 by RT-qPCR or western blot, respectively. (C and D) SCC13 cells were transfected with virus encoding control shRNA (shCtrl) or sh*IRF1* for 72 h, and harvested for analyzing the relative mRNA (C) and protein (D) level of PD-L1 by RT-qPCR and western blot, respectively. (E and F) SCC13 cells were transfected with empty vectors (Vec) or TBX2-DN (dominant negative)-encoding plasmids for 48 h and treated with vehicle or 20 ng/ml IFNγ for 1 h, then cells were harvested for determining the relative protein level of pSTAT1 (E and F), STAT1 (E), and IRF1 (E and G) by western blot. $n = 4$. *, the significance compared to control was analyzed by one-way ANOVA test.

expression level of IRF1 in SCC13 cells, and the results of gene expression and protein expression levels were consistent with the trend of PD-L1 (Figure 4A,B). Using shRNA to knock down the expression of IRF1 also downregulated the relative gene (0.17 ± 0.05) and protein (0.23 ± 0.05) expression levels of PD-L1 (Figure 4C,D). The above results suggest that TBX2 may regulate the expression of PD-L1 by regulating the expression of IRF1. By treating the cells with IFNγ, pSTAT1 level in Vec-transfected cells was elevated significantly. It was found that TBX2-DN-transfected cells, in which TBX2 had no function, activation of IFNγ signal was not affected, which was indicated by pSTAT1 expression (Figure 4E,F). However, it affected the protein expression level of IRF1 (Figure 4G) (without IFNγ: 1.00 ± 0.23 vs. 0.23 ± 0.04; with IFNγ: 3.67 ± 0.50 vs. 0.27 ± 0.06). It suggested that TBX2 did not participate in the activation of IFNγ signal, but it mediated the expression of IRF1, which is the downstream of IFNγ.

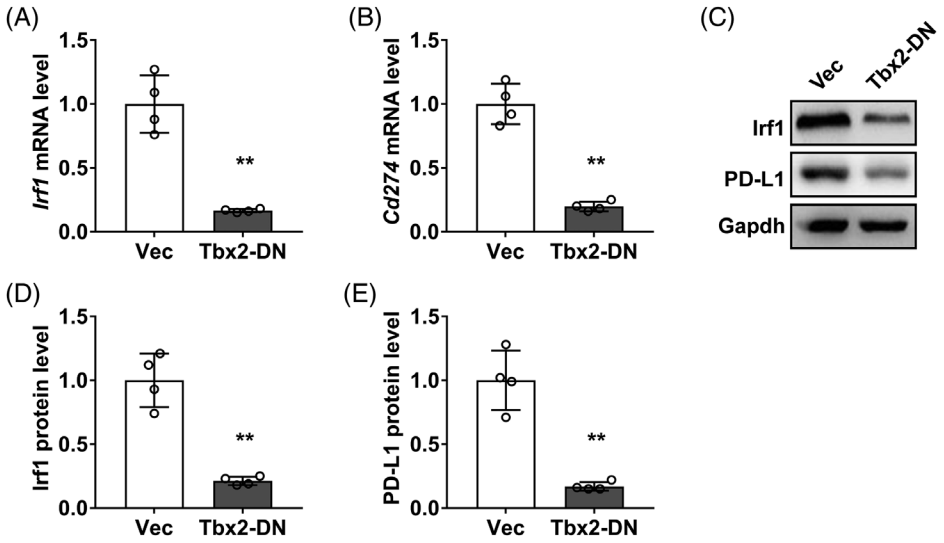

FIGURE 5 Tbx2-Irf1 axis mediated the expression of programmed death ligand 1 (PD-L1) in murine cutaneous squamous cell carcinoma (CSCC) cells. PDV cells were transfected with empty vectors (Vec) or Tbx2-DN (dominant negative)-encoding plasmids for 48 h and harvested for analyzing the relative mRNA (A and B) and protein (C–E) level of Irf1 (A, C, and D) and PD-L1 (Cd274) (B, C, and E) by RT-qPCR or western blot, respectively. $n = 4$. *, the significance compared to control was analyzed by two-tailed *t*-test.

We also tested in murine CSCC cells with PDV cell line. We verified that in the murine CSCC cell line PDV cells, overexpression of Tbx2-DN also inhibited the mRNA expression level of *Irf1* gene (0.17 ± 0.01) (Figure 5A) and PD-L1 gene *Cd274* (0.20 ± 0.04) (Figure 5B), as well as for protein expression level (Irf1: 0.21 ± 0.03; PD-L1: 0.17 ± 0.03) (Figure 5C–E). Therefore, in murine CSCC cells, Tbx2 also affected the expression of Irf1, which could regulate the expression level of PD-L1.

3.5 | Tbx2 promoted the progression of PDV tumor via inhibiting the infiltration of T cells in mice

Finally, in order to explore the function of Tbx2 in the progression of CSCC in vivo, we established a PDV xenograft tumor model with overexpression of Tbx2-DN. We found that overexpression of Tbx2-DN significantly inhibited tumor progression, the growth rate of tumor volume and endpoint tumor weight (1.90 ± 0.35 g vs. 0.53 ± 0.25 g) were both significantly lower than Vec-transfected xenografts (Figure 6A,B). In addition, the gene expression levels (*Irf1*: 0.39 ± 0.13; *Cd274*: 0.38 ± 0.15) (Figure 6C,D) and protein expression levels (Irf1: 0.42 ± 0.11; PD-L1: 0.40 ± 0.13) (Figure 6E–G) of Irf1 and PD-L1 in Tbx2-DN tumor tissues were also significantly reduced, indicating that Tbx2 also regulated the expression of Irf1 in xenograft tumors.

In order to further explore whether Tbx2 mediates tumor progression through regulating the antitumor immune response, the immune microenvironment in the transplanted tumor model was tested. The FACS analysis results showed that the proportion of $CD3^+$ (22.44 ± 3.62 vs. 43.64 ± 11.63) and $CD8^+$ T (15.45 ± 3.40 vs. 31.21 ± 9.35) cells in Tbx2-DN xenograft tumors increased (Figure 7A–C), while the proportion of PD-1^+ cells in $CD8^+$ T cells decreased (proportion: 41.50 ± 6.98 vs. 22.32 ± 10.35; Mean Fluorescence Intensity (MFI): 69.13 ± 19.70 vs. 34.36 ± 16.64) (Figure 7D–F). These results suggested that the immuno infiltration of Tbx2-DN tumors accelerated the exhausting of PD-1^+ T cells in xenograft tumors.

4 | DISCUSSION

In this study, by analyzing the gene profile from CSCC patient tumors and proof from several CSCC cell lines, as well as murine PDX CSCC model, we identified TBX2 as an important factor that can regulate the immune-response for PD-1 therapy. TBX2 functions mainly as a transcriptional repressor, playing a role in cell cycle progression. Previous research has indicated that it may be an immortalizing agent that plays a potential role in tumorigenesis.[16] In nonhematopoietic cell lines, TBX2 has also been found that it can induce endogenous IFNγ transcription.[17] Thus, TBX2 is not surprising to be found overexpressed in CSCC.

The IFNγ signaling pathway is important in tumorigenesis and progression and can exert its killing effect directly by inducing apoptosis or promoting nonapoptotic cell death, or indirectly by making cancer cells vulnerable to apoptosis-inducing immune responses or chemotherapy.[18,19] It is reported that IFNγ can induce IRF1 (a tumor suppressor gene) thus represses Bcl2 and promotes Bak expression.[20] Subsequently, these signals promote cytochrome c release from mitochondria together with activation of caspases, leading to tumor cell apoptosis.[21] The upregulation of IRF1 in CSCC cells requires the mediation of TBX2, which resists T cell recognition and killing by upregulating PD-L1 via IRF1. IFNγ is mainly produced by T cells, natural killer (NK) cells and NK T cells,[21] if CD8+ T cells inhibit tumor

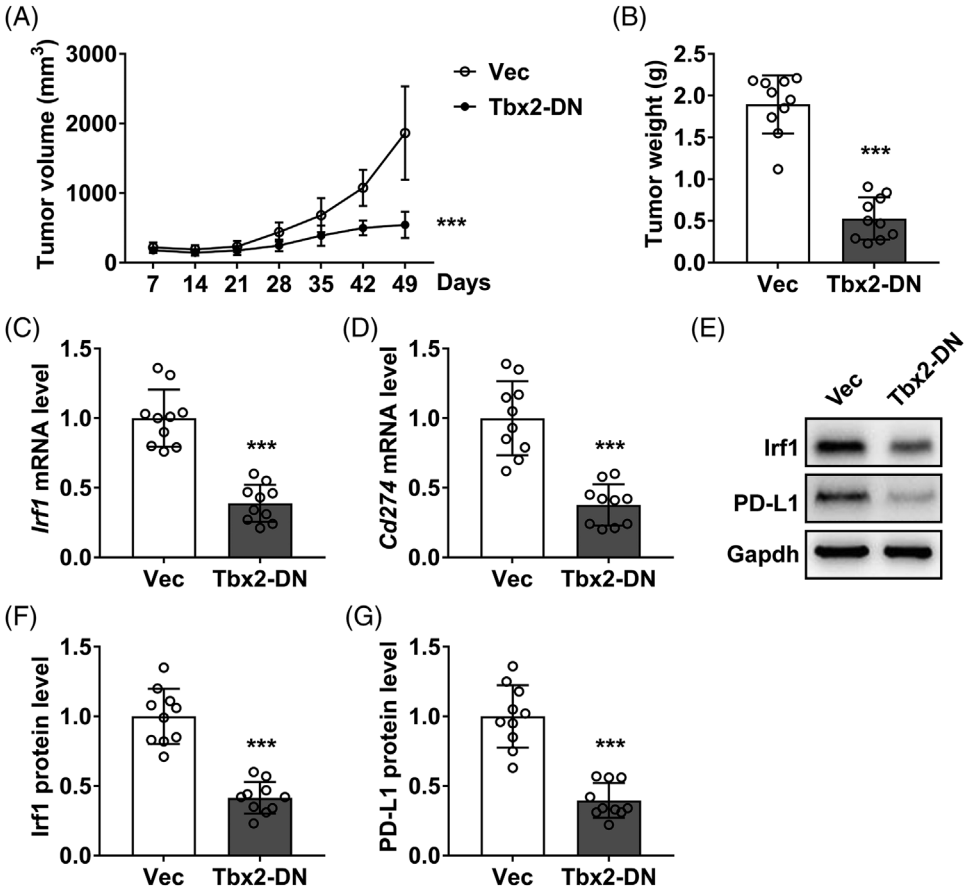

FIGURE 6 Tbx2 promoted the progression of PDV tumor in mice. PDV cells were transduced with empty vectors (Vec) or Tbx2-DN (dominant negative)-encoding lenti virus and selected for stable expression and seeded to mice subcutaneously on back (1×10^7 cells / mouse). (A) Tumor volume was measured at indicated time points. $n = 10$. *, the significance compared to control was analyzed by two-way ANOVA test. (B) Tumor weight was determined after mice sacrifice at the end point (day 49). (C–G) Tumors were harvested after mice sacrifice at the end point (day 49) to prepared RNA and protein sample for analyzing the relative mRNA (C and D) and protein (E–G) level of Irf1 (C, E, and F) and programmed death ligand 1 (PD-L1) (Cd274) (D, E, and G) by RT-qPCR or western blot, respectively. $n = 10$. *, the significance compared to control was analyzed by two-tailed *t*-test.

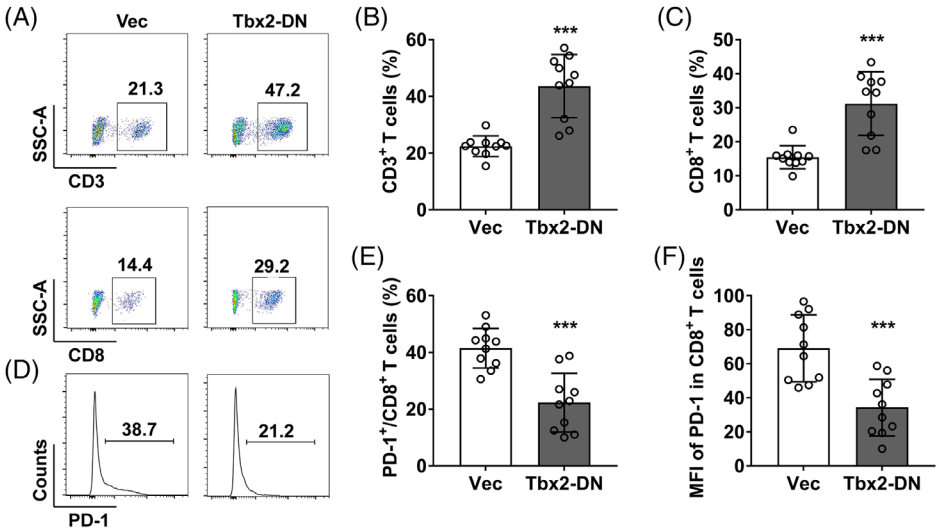

FIGURE 7 Tumor expressed Tbx2 inhibited the infiltration of T cells into tumors in mice. Tumors were harvested after mice sacrifice at the end point (day 49) to prepared cell suspension for analyzing the percentage of $CD3^+$ (A and B), $CD8^+$ (A and C) T cells, **programmed death ligand 1^+ (PD-$L1^+$)** in CD8 T cells (D and E), and the MFI of PD-1 in CD8 T cells (F) by FACS. $n = 10$. *, the significance compared to control was analyzed by two-tailed *t*-test.

growth by producing IFNγ, and at the same time, we can also inhibit or downregulate TBX2, IFNγ-induced downregulation of IRF1 can be promoted, thereby downregulate PD-L1, resulting in promoting T cell killing. Previous studies have shown that the dormant state of tumors can determine whether IFNγ can suppress dormant cells or edit dormant tumor cells, leading to tumor recurrence.[22] In particular, Ki67-low indolent cancer cells are vulnerable to immunomodulation that can evade immunotherapy, whereas Ki67-quiescent dormant cells are not immune-modulated and thus remain dormant by IFNγ-producing T cells.[23] IFNγ can stimulate the PD-L1 expression in Ki67 low indolent cells and activate STAT1 to induce the PD-L1 expression in cancer cells.[24,25] If TBX2 can be inhibited at the same time, it will not affect the activation of IFNγ, STAT1 remains functioning downstream effects but can inhibit the expression of PD-L1 by repressing IRF1, thus synergistically promote the killing effect of T cells. Therefore, TBX2 is a potential target for synergistic immunotherapy with PD-1 antibody drug. The development of drugs targeting TBX2 will boost the effect of immunotherapy in many cancers including metastatic malignant CSCC.

5 | CONCLUSIONS

TBX2 mediates antitumor immune response in CSCC by regulating the expression of PD-L1 through IRF1. It might be a prognostic marker in CSCC and synergistic target for PD-1 immunotherapy.

ACKNOWLEDGMENT

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

FUNDING INFORMATION

Class B of Qingdao Medical and Health Key Discipline Construction Project, Grant Number: 2020–2023

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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