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Original Research Article

Knockdown of PLAGL2 inhibits oral squamous cell carcinoma cell growth and cisplatin resistance via ZEB1/PD-L1 pathway

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Abstract

Purpose: *To investigate the effects of pleomorphic adenoma gene like-2 (PLAGL2) on oral squamous cell carcinoma (OSCC).*
Methods: *The effects of PLAGL2 on OSCC (CAL-27 and HSC-3) were conducted with loss- and gain-functional assays. Cell proliferation was determined by Cell Counting Kit-8 (CCK8) and colony formation assays. Cell metastasis was assessed by Transwell and wound healing assays. Cell apoptosis of cisplatin-resistant OSCC was evaluated by flow cytometry.*
Results: *PLAGL2 expression was significantly elevated in OSCC ($p < 0.001$), while silencing of PLAGL2 significantly reduced cell proliferation and metastasis of OSCC ($p < 0.001$). However, overexpression of PLAGL2 promoted OSCC progression through increase in cell proliferation, invasion, and migration. Knockdown of PLAGL2 promoted cell apoptosis of -resistant OSCC, but significantly downregulated protein expression of programmed cell death ligand 1(PD-L1) and zinc finger E-box-binding homeobox 1 (ZEB1) in OSCC ($p < 0.01$). Moreover, loss of ZEB1 attenuated the PLAGL2 overexpression-induced increase in ZEB1 and PD-L1. Loss of PLAGL2 also reduced in vivo tumor growth of OSCC via regulation of cluster of differentiation 4 protein (CD4) and CD8.*
Conclusion: *Knockdown of PLAGL2 exerts anti-tumor effects, improves cisplatin resistance, and enhances anti-tumor immunity in OSCC through suppression of ZEB1/PD-L1 pathway. Thus, PLAGL2 might be a potential therapeutic target for the treatment of OSCC.*

Keywords: *PLAGL2, Oral squamous cell carcinoma (OSCC), Cell proliferation, Metastasis, Tumor immunity, ZEB1, Programmed cell death ligand 1*

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is a subtype of head and neck cancer, and its incidence and prevalence have increased significantly over the last 20 years [1,2]. Surgery, chemoradiation, and radiation therapies are widely used in the treatment of OSCC. However, tumor recurrence and metastasis contribute to the poor prognosis of OSCC [3]. Therefore, more effective therapies are needed for the improvement of OSCC outcomes. Immune checkpoints, such as programmed cell death ligand 1 (PD-L1) and programmed cell death

protein 1 (PD1), facilitate the escape of tumor cells from intrinsic immune detection [4]. Extrinsic factors, epigenetic alterations, genomic alterations, stem cell signals, constitutive oncogenic signals, and inducers of epithelial-to-mesenchymal transition such as zinc finger E-box-binding homeobox 1 (ZEB1), stimulate PD-L1 expression in tumor cells [5]. PD-L1 is expressed on the surface of tumor cells, and contributes to chemoresistance, tumor invasion, epithelial-to-mesenchymal transition, and cancer stemness in distinct tumors, thus suppressing the anti-tumor immune response [5]. PD-L1 is upregulated in OSCC, predicts poor prognosis, and exhibits immunosuppressive activity [6]. Likewise, ZEB1 promotes cisplatin resistance (CDDP-R) of cancer cells [7]. However, the role of ZEB1/PD-L1 in tumor progression, immunity, and drug resistance of OSCC remains unclear.

Pleomorphic adenoma gene like-2 (PLAGL2) is a zinc finger protein, functions as a transcription factor involved in tumorigenesis of distinct cancers [8]. Overexpression of PLAGL2 induces proliferation and metastasis of acute myeloid leukemia [9]. Moreover, the epithelial-mesenchymal transition of colorectal adenocarcinoma is enhanced by PLAGL2 [10], and PLAGL2 stimulate metastasis and the epithelial-mesenchymal transition of colorectal cancer through up-regulation of ZEB1 [11]. PLAGL2 is also associated with immune cell infiltration in glioblastoma multiform and regulates the tumor immunity [12]. Pleomorphic adenoma gene like-2 also decreases the response of hepatocellular carcinoma cells to erlotinib [13]. Therefore, PLAGL2 might contribute to growth, tumor immunity and drug resistance of OSCC through regulation of the ZEB1/PD-L1 pathway. In this study, the effects of PLAGL2 on cell proliferation, metastasis, and tumor immunity of OSCC were investigated.

EXPERIMENTAL

Bioinformatic analysis

Expression of *PLAGL2* in OSCC was investigated in TIMER: Tumor Immune Estimation Resource (https://cistrome.shinyapps.io/timer/) and the University of Alabama at Birmingham Cancer data analysis Portal (UALCAN; http://ualcan.path.uab.edu/).

Cell culture

Human OSCC cells (HSC-3, CAL-27, SCC-9, and SCC-25) and oral keratinocytes (HOK) (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 media (Gibco, Carlsbad, CA, USA) containing 10 % fetal bovine serum (Thermo Fisher, Waltham, MA, USA). To establish chemoresistant OSCC cell sublines, HSC-3 and CAL-27 cells were treated with increasing doses of cisplatin (CDDP) i.e. 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 μg/mL every two weeks. The IC_{50} values of HSC-3, CAL-27, HSC-3 CDDP-R, and CAL-27 CDDP-R cells were determined using the Cell Counting Kit-8 (CCK8) assay.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The RNAs were isolated from the cells using Trizol agent (Sigma-Aldrich, St. Louis, MO, USA) and then reverse-transcribed into cDNAs using PrimeScript RT Reagent (Takara, Shiga, Japan). The mRNA expression of *PLAGL2* was determined using SYBR Green Master Mix (Roche, Mannheim, Germany) and analyzed using the $2^{-\Delta\Delta Cq}$ method. GAPDH was used as endogenous control. Primers were shown in Table 1.

Table 1: Primers for RT-qPCR

	forward	reverse
PLAGL2	5'-GAGTCAAGTGAAGTGCCAATGT-3'	5'-TGAGGGCAGCTATATGGTCTC-3'
GAPDH	5'-ACCACAGTCCATGCCATCAC-3'	5'-TCCACCACCCTGTTGCTGTA-3'

Cell viability and proliferation assays

The HSC-3, CAL-27, HSC-3 CDDP-R, and CAL-27 CDDP-R cells were seeded in 96-well plates and treated with 0.5, 1, 2, 4, 8, 16, and 32 μM CDDP for 24 h. Cells were then incubated with CCK8 (Dojindo, Tokyo, Japan) for 2 h, and absorbance at 450 nm was measured with a Microplate Autoreader (Thermo Fisher) to determine IC_{50} values. HSC-3 and CAL-27 cells were also seeded in 96-well plates and transfected with sh-PLAGL2#1, sh-PLAGL2#2, pcDNA-PLAGL2, sh-NC, or pcDNA (GenePharma, Suzhou, China) using Lipofectamine 2000 (Gibco) for 24 h. Cells were grown for another 24, 48, or 72 h and then incubated with CCK8 (Dojindo, Tokyo, Japan) for 2 hours. Absorbance at 450 nm was measured with a Microplate Autoreader (Thermo Fisher). To evaluate cell proliferation, HSC-3 and CAL-27 cells were seeded in 6-well plates and cultured in the media for 10 days. The cells were fixed with 4% paraformaldehyde and then stained with crystal violet. The number of colony-forming cells

was calculated under a microscope (Olympus, Tokyo, Japan).

Transwell and wound healing assays

HSC-3 and CAL-27 cells in serum-free RPMI-1640 media were plated into the upper chamber of Matrigel-coated wells (Corning, Tewksbury, MA, USA), while the lower chamber was filled with media containing 20 % fetal bovine serum. Twenty-four hours later, the invasive cells in the lower chamber were fixed in methanol and stained with crystal violet. The number of invasive cells was calculated under the microscope. For wound healing, HSC-3 and CAL-27 cells were seeded in 6-well plates. A pipette tip was used to generate scratches in the media of plates. Twenty-four hours later, the wound widths were calculated under the microscope.

Flow cytometry

HSC-3 CDDP-R and CAL-27 CDDP-R cells were transfected with sh-PLAGL2#1, sh-PLAGL2#2, pcDNA-PLAGL2, sh-NC, or pcDNA for 48 h. The cells were harvested and then double-stained with propidium iodide and Annexin V-FITC. The cells were analyzed on a FACScan flow cytometer (BD Biosciences; San Diego, CA, USA).

Mouse xenograft assay

Eight female BALB/c nude mice (18-22 g and 4-6 weeks old) were divided into two groups: sh-PLAGL2 (n=4) and sh-NC (n=4). The study was approved by the Ethics Committee of Wuhan Hankou Hospital (approval no. hyll2021113) and conducted in accordance with the National Institutes of Health (NIH) Laboratory Animal Care and Use Guidelines [14]. shRNAs (sh-PLAGL2#2 and sh-NC) were packaged in lentivirus from Genepharma (Shanghai, China) and then used to infect CAL-27 cells to generate cells with stable knockdown of *PLAGL2*. CAL-27 cells were then subcutaneously injected into the nude mice. Tumor volume was calculated every 10 days. Tumors were isolated and weighted 40 days after the injection.

Immunohistochemistry

Tumor tissues were fixed with 10 % formalin overnight and embedded in paraffin. The tissues were sliced into 4 μm thick sections. Sections were treated with 3 % hydrogen peroxide followed by dewaxing and rehydration and then immersed in Tris-EDTA buffer containing 0.05 % Tween 20. Sections were blocked in 5 % dry milk and incubated overnight with antibodies: anti- Ki-67 (1:80), anti-CD4 (1:50), and anti-CD8 (1:100). Lastly, sections were incubated in horseradish peroxidase-conjugated secondary antibody and examined under the microscope.

Western blot

Tissues and cells were lysed in RIPA buffer (Beyotime, Beijing, China), and the protein samples were segregated using SDS-PAGE. The samples were then transferred onto nitrocellulose membranes, and the membranes blocked in 5 % dry milk. The membranes were incubated with primary antibodies: anti-PLAGL2 and anti-GAPDH (1:2000), anti-E-cadherin and anti-N-cadherin (1:2500), and anti-PD-L1 and anti-ZEB1 (1:3000). The membranes were then incubated with secondary antibodies (1:4000) and subjected to a chemiluminescence reagent kit (Beyotime). All the antibodies were purchased from Abcam (Cambridge, MA, USA).

Statistical analysis

All data are shown as mean ± standard error of the mean and analyzed by Student's t test or one-way analysis of variance using SPSS 20.0 software (SPSS Inc, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Upregulation of *PLAGL2* in OSCC

Data from TIMER (Figure 1 A) and UALCAN (Figure 1 B) indicated that *PLAGL2* was significantly upregulated in OSCC tissues when compared to normal tissues. Human OSCC cells (HSC-3, CAL-27, SCC-9, and SCC-25) also had higher expression of *PLAGL2* than HOK cells (Figures 1 C and D). The HSC-3 and CAL-27 cells had the highest *PLAGL2* expression among the OSCC cells and were subjected to functional assays.

PLAGL2 contributed to OSCC proliferation

HSC-3 and CAL-27 cells were transfected with shRNAs or pcDNA vectors to decrease or increase the protein expression of PLAGL2 (Figure 2 A). Cell viability of HSC-3 and CAL-27 cells was enhanced by overexpression of *PLAGL2*, whereas it was reduced by silencing *PLAGL2* (Figure 2 B). Overexpression of *PLAGL2* increased the number of colonies, whereas silencing of *PLAGL2* decreased the numbers in HSC-3 and CAL-27 cells (Figure 2 C).

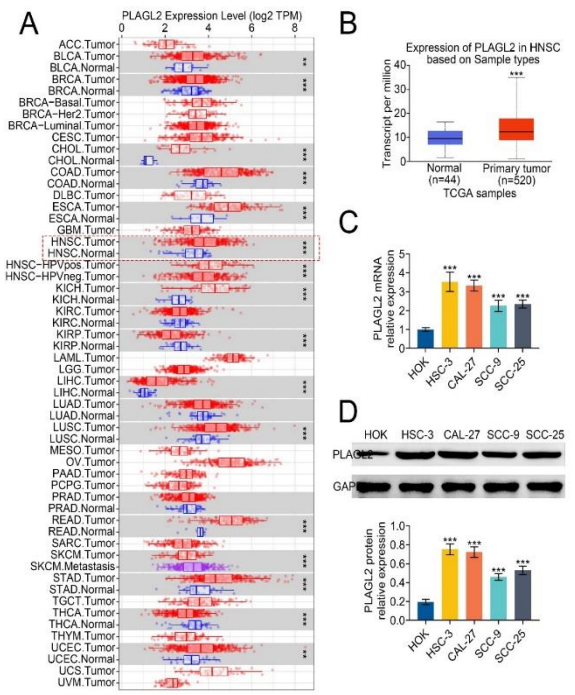

Figure 1: Upregulation of *PLAGL2* in OSCC (A) *PLAGL2* was significantly upregulated in OSCC tissues compared to normal tissues based on TIMER database. (B) PLAGL2 was significantly upregulated in OSCC tissues compared to normal tissues based on the UALCAN database. (C) mRNA expression of *PLAGL2* was upregulated in human OSCC cells (HSC-3, CAL-27, SCC-9, and SCC-25). (D) Protein expression of PLAGL2 was upregulated in human OSCC cells (HSC-3, CAL-27, SCC-9, and SCC-25). ***P < 0.001

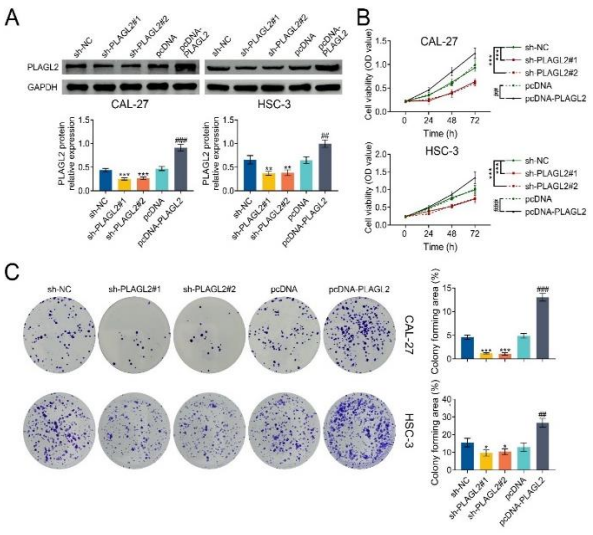

Figure 2: *PLAGL2* contributed to OSCC cell proliferation. (A) HSC-3 and CAL-27 cells were transfected with shRNAs or pcDNA vector to decrease or increase protein expression of PLAGL2. (B) Overexpression of *PLAGL2* increased cell viability, whereas silencing of *PLAGL2* decreased cell viability in HSC-3 and CAL-27 cells. (C) Overexpression of *PLAGL2* increased the number of colonies, whereas silencing of *PLAGL2* decreased the number of colonies in HSC-3 and CAL-27 cells. ##P < 0.01, ###p < 0.001 vs. pcDNA. ***p < 0.001 vs. sh-NC

PLAGL2 contributed to OSCC cell metastasis

Overexpression of *PLAGL2* enhanced the number of invasive cells, whereas silencing of *PLAGL2* decreased the number of these cells (Figure 3 A). The migration of HSC-3 and CAL-27 cells was suppressed by silencing *PLAGL2* but promoted by overexpression of *PLAGL2* (Figure 3 B). Protein expression of E-cadherin was upregulated, whereas N-cadherin was downregulated in HSC-3 and CAL-27 cells by knockdown of *PLAGL2* (Figure 4). However, overexpression of *PLAGL2* demonstrated opposite effects on E-cadherin and N-cadherin expression (Figure 4).

PLAGL2 contributed to cisplatin resistance of OSCC

HSC-3 and CAL-27 cells were treated with CDDP by dose escalation to induce resistance. IC_{50} values of HSC-3 CDDP-R and CAL-27 CDDP-R cells were higher than those of HSC-3 and CAL-27 cells (Figure 5 A). Overexpression of *PLAGL2* reduced cell apoptosis of HSC-3 CDDP-R and CAL-27 CDDP-R cells (Figure 5 B and C). However, loss of *PLAGL2* promoted apoptosis of HSC-3 CDDP-R and CAL-27 CDDP-R cells (Figure 5 B and C).

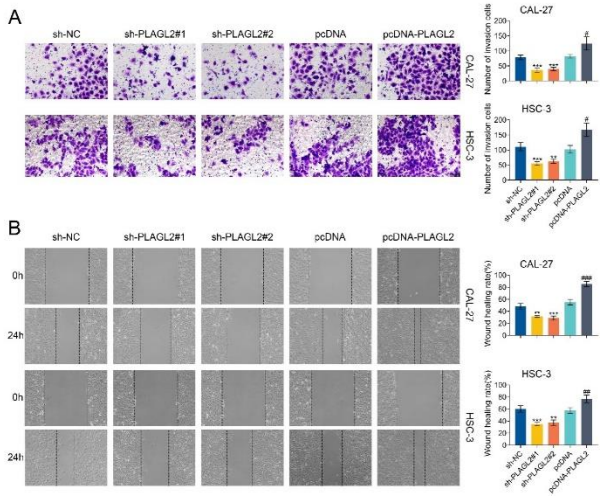

Figure 3: *PLAGL2* contributed to OSCC cell metastasis. (A) Overexpression of *PLAGL2* increased the number of invasive cells, whereas silencing of *PLAGL2* decreased the numbers in HSC-3 and CAL-27 cells. (B) Overexpression of *PLAGL2* increased cell migration, whereas silencing of *PLAGL2* decreased migration in HSC-3 and CAL-27 cells. #P < 0.05, ##p < 0.01, ###p < 0.001 vs. pcDNA; **p < 0.01, ***p < 0.001 vs. sh-NC

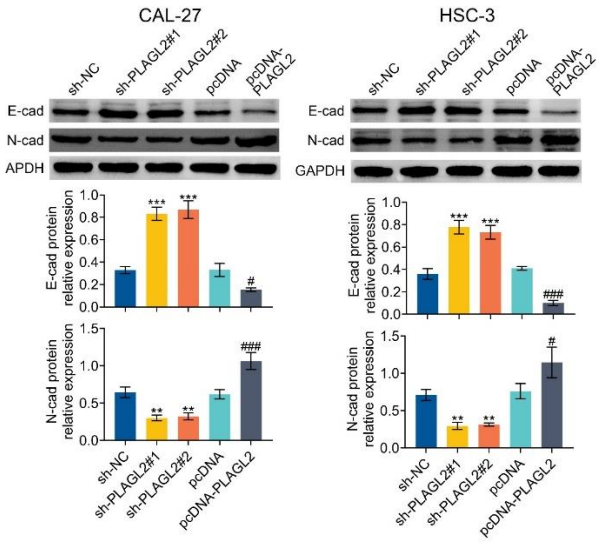

Figure 4: Overexpression of *PLAGL2* increased N-cadherin and decreased E-cadherin in HSC-3 and CAL-27 cells, whereas silencing of *PLAGL2* decreased N-cadherin and increased E-cadherin in HSC-3 and CAL-27 cells. #*P* < 0.05, ###*p* < 0.001 vs. pcDNA. ***p* < 0.01, ****p* < 0.001 vs. sh-NC

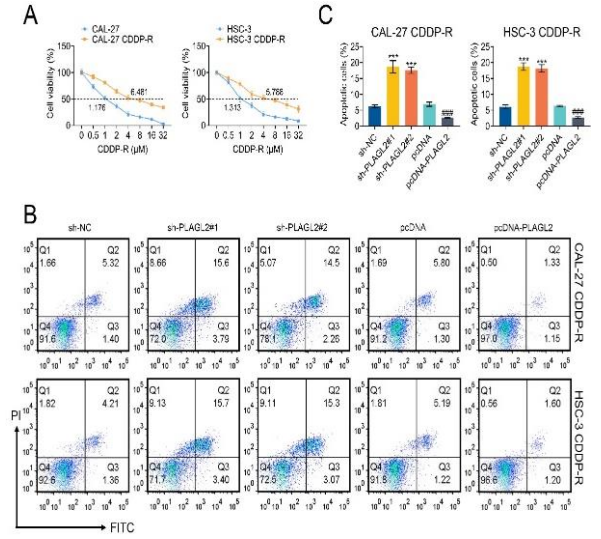

Figure 5: *PLAGL2* enhanced cisplatin resistance of OSCC. (A) IC50 values of HSC-3 CDDP-R and CAL-27 CDDP-R cells were higher than that of HSC-3 and CAL-27 cells. (B) Overexpression of *PLAGL2* reduced cell apoptosis of HSC-3 CDDP-R and CAL-27 CDDP-R cells, whereas loss of *PLAGL2* promoted apoptosis of HSC-3 CDDP-R and CAL-27 CDDP-R cells. © Relative apoptotic cells of HSC-3 CDDP-R and CAL-27 CDDP-R cells. ###*P* < 0.001 vs. pcDNA; ****p* < 0.001 vs. sh-NC

PLAGL2 mediated ZEB1/PD-L1 pathway in OSCC

Overexpression of *PLAGL2* enhanced protein expression of ZEB1 and PD-L1, whereas silencing of *PLAGL2* reduced the expression of these proteins (Figure 6 A). Knockdown of *ZEB1* attenuated the *PLAGL2*-induced increase of ZEB1 and PD-L1 (Figure 6 B), suggesting that *PLAGL2* contributed to activation of the ZEB1/PD-L1 pathway in OSCC.

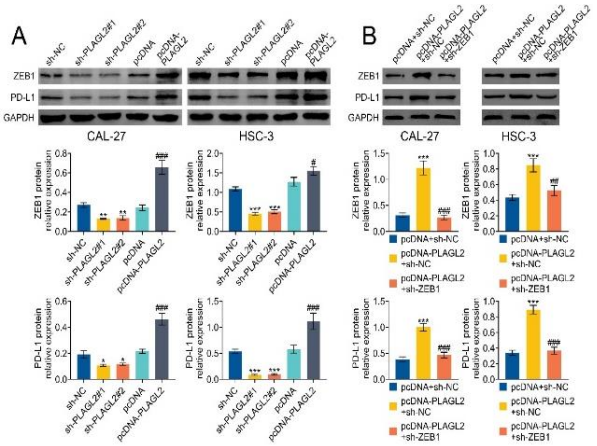

Figure 6: *PLAGL2* mediated ZEB1/PD-L1 pathway in OSCC. (A) Overexpression of *PLAGL2* enhanced protein expression of ZEB1 and PD-L1, whereas silencing of *PLAGL2* reduced the expression in HSC-3 and CAL-27 cells. (B) Knockdown of *ZEB1* attenuated the *PLAGL2*-induced increase of ZEB1 and PD-L1 in HSC-3 and CAL-27 cells. # *p* < 0.05, ## *p* < 0.01, ### *p* < 0.001 vs. pcDNA. * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001 vs. sh-NC

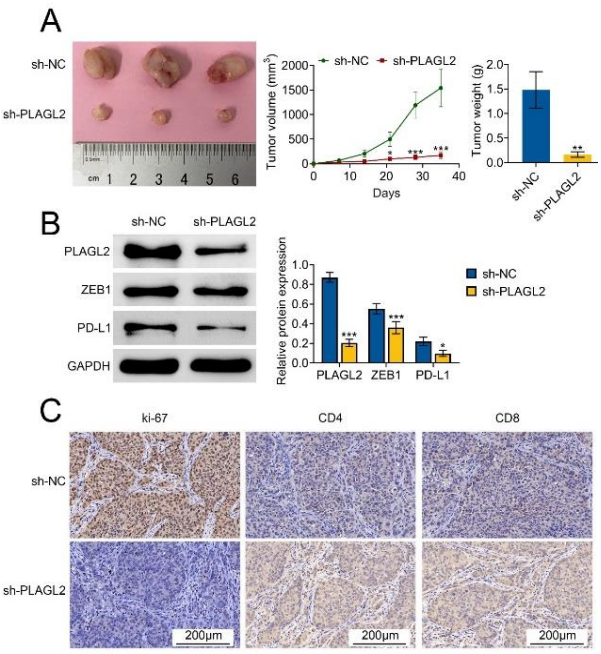

Figure 7: *PLAGL2* mediated tumor immunity in OSCC. (A) Silencing of *PLAGL2* reduced tumor volume and weight to inhibit the *in vivo* tumor growth of OSCC. (B) Knockdown of *PLAGL2* downregulated protein expression of ZEB1 and PD-L1 in tumor tissues. © Loss of *PLAGL2* reduced ki-67 expression and enhanced CD4 and CD8 in OSCC tissues. **P* < 0.05, ***p* < 0.01, ****p* < 0.001 vs. sh-NC

PLAGL2 mediated tumor immunity in OSCC

In vivo tumor growth of OSCC was suppressed by silencing of *PLAGL2*, including reduced tumor volume and weight (Figure 7 A). Knockdown of *PLAGL2* downregulated protein expressions of ZEB1 and PD-L1 in the tumor tissues (Figure 7 B). Immunohistochemistry indicated that loss of *PLAGL2* reduced ki-67 expression and enhanced CD4 and CD8 expression (Figure 7 C), thus promoting anti-tumor immunity in OSCC.

DISCUSSION

Pleomorphic adenoma gene like-2 is positively correlated with infiltration of immune cells, neutrophils, macrophages, $CD4^+$ T cells, and B cells in glioblastoma multiform, thereby participating in the modulation of tumor immunity [12]. Moreover, *PLAGL2* exerts suppressive or carcinogenic activities in distinct tumors [12]. This study found that *PLAGL2* functioned as an oncogene in OSCC and reduced immunosuppressive effects through activation of ZEB1/PD-L1 signaling.

The upregulation of *PLAGL2* in OSCC was initially identified based on bioinformatic analysis. Knockdown of *PLAGL2* reduced cell proliferation and metastasis of OSCC. Pleomorphic adenoma gene like-2 expression is negatively associated with the expression of E-cadherin and inversely related to β-catenin, Vimentin, and N-cadherin in patients with colorectal cancer [11]. Overexpression of *PLAGL2* promote the epithelial-mesenchymal transition through reduce of E-cadherin and enhance of N-cadherin and vimentin [11]. This study showed that the silencing of *PLAGL2* reduced N-cadherin and enhanced E-cadherin to inhibit the epithelial-mesenchymal transition of OSCC.

Previous studies showed that up-regulation of PD-L1 reduced anti-tumor immunity of OSCC had [15], and blockade of PD-L1 enhanced anti-tumor immunity in these cells [16]. ZEB1 functions as an activator of the epithelial-mesenchymal transition and relieves the repression of miR-200 by PD-L1 in lung cancer cells, thus promoting immunosuppression of $CD8^+$ T cells and metastasis of lung cancer [17]. Suppression of the ZEB1/PD-L1 pathway inhibits immune escape and tumor progression of colorectal cancer [18]. Pleomorphic adenoma gene like-2 decreases phosphorylation of β-catenin and enhances nuclear translocation to up-regulate ZEB1 [11]. In this study, overexpression of *PLAGL2* enhanced ZEB1 and PD-L1 expression, and knockdown of ZEB1 attenuated the *PLAGL2*-induced increase of ZEB1 and PD-L1 in OSCC. This suggests that *PLAGL2* promotes PD-L1 expression and contributes to immune escape and tumor progression in OSCC through upregulation of ZEB1. Moreover, silencing of *PLAGL2* downregulated protein expression of ZEB1 and PD-L1, whereas it enhanced CD4 and CD8 to inhibit *in vivo* immune escape and tumor growth of OSCC. However, the effects of *PLAGL2* on *in vivo* secretion of cytokines and proportions of $CD4^+$ T, $CD8^+$ T cells in OSCC remain unknown.

CONCLUSION

The findings of this study show that *PLAGL2* functions as an oncogene in OSCC by promoting cell proliferation, invasion and migration, and epithelial-mesenchymal transition. Moreover, knockdown of *PLAGL2* enhances anti-tumor immunity by targeting ZEB1 to reduce PD-L1 expression. Therefore, this study suggests a potential *PLAGL2* is a potential target for immune modulation and treatment of OSCC. However, the effects of PD-L1 inhibitors and *PLAGL2* suppression in OSCC should be investigated in further research.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Changqing Zhang and Fei Ye designed the studies and carried them out, supervised the data collection,

analyzed the data, interpreted the data, prepared the manuscript for publication, and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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