

Original Research

Silencing PLOD2 attenuates cancer stem cell-like characteristics and cisplatin-resistant through Integrin β 1 in laryngeal cancer

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ABSTRACT

Laryngeal cancer (LC) is an aggressive malignancy resistant to drug treatments. It has been postulated that cancer stem cells (CSCs) persist in a unique population of cancer cells involved in tumor progression and drug-resistance. In the present study, the effects of PLOD2 expression on ordinary and Cisplatin (DDP)-resistance (R) cells were investigated in TU686 and TU138 cells and Xenograft model. Cell viability, invasion and cell apoptosis, CD44 and CD133 expressions, MRP1 and P-gp expressions were measured by CCK-8 assay, Transwell, flow cytometry, immunofluorescence and Western blotting respectively. The results of our study demonstrated that suppressing the expression of PLOD2 could mediate LC stem cell-like features by decrease cell viability and invasion, increase apoptotic rate, decrease CD44 and CD133 expressions via Integrin β 1. Meanwhile, the inhibition of PLOD2 expression could decrease P-gp and MRP1 expression thus markedly regulate DDP-R LC cells stemness and drug-resistance via Integrin β 1. Our findings provided a new rationale for subsequent academic and clinical research on LC drug-resistance.

Introduction

Head and neck squamous cell carcinomas (HNSCCs), which is arising from the oral cavity mucosa, larynx and pharynx, is the most primary head and neck malignancies and has high incidence and mortality rate [1,2]. Laryngeal cancer (LC) is a common malignancy which represents one-third of all HNSCCs and remains a significant source of morbidity and mortality. With the comprehensive development of treatment measures and diagnostic strategies, surgical techniques in laryngeal carcinomas have evolved greatly, partial surgery even transoral robotic surgery has become increasingly used to manage laryngeal and pharyngeal cancers [3–5]. Even the survival rate was increased, there was still about 30–40% of LC patients died of tumor recurrence or metastasis. Due to the high rates of metastasis and recurrence and resistance to traditional chemotherapy, the patients still present a poor prognosis. Hence, identify predictive biomarkers and find most effective and least toxic therapies will promise new avenue for laryngeal cancers therapy.

Previous studies revealed that Cancer stem cells (CSCs) refer to a

unique population of cancer cells involved in tumor initiation, progression, drug-resistance and relapse [6,7]. A series of CSC-specific surface biomarkers including CD9, CD24, CD44, and CD133 have been reported to be regarded as reliable means of identifying CSCs [8,9]. CSCs are known to be involved in LC clonogenicity core and play a vital role in LC dissemination, drug-resistance and recurrences poor prognosis [10]. According to literature data cisplatin and cetuximab resistance in CSCs from HNSCC lines that had high expression of the CD44 biomarker [11, 12].

Tumor diffusion and metastasis are important causes of tumor deterioration and the process was associated with several genes and proteins such as Integrin β 1 and PLOD2 [13–15]. Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2), also known as lysyl hydroxylase 2 (LH2), is a member of the PLOD family. Accumulating evidence indicates that PLOD2 is essential enzyme in the lysyl hydroxylation of collagen molecules [16,17]. Furthermore, numerous studies have demonstrated that PLOD2 expression was associated with several types of cancers such as HNSCCs, colorectal cancer (CRC), oral squamous cell carcinoma (OSCC), hepatocellular carcinoma and pancreatic cancer

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[18]. Knockdown of PLOD2 attenuated CRC cell proliferation, migration, and invasiveness via regulating the expression of hexokinase 2, suggesting PLOD2 as a promising therapeutic target for CRC treatment [19]. Studies also revealed that PLOD2 was a poor prognostic biomarker for OSCC patients and may affect the metastasis of OSCC through EMT pathway [16]. Integrin $\beta 1$ is an important member of transmembrane glycoproteins and is found to be associated with cancer metastasis [20]. More interestingly, studies revealed that PLOD2 directly catalyzes the hydroxylation of Integrin $\beta 1$ and leads to increased cancer invasion and metastasis [21]. Studies shown that PLOD2-driven IL-6/STAT3 signaling promotes the invasion and metastasis of oral squamous cell carcinoma via activation of Integrin $\beta 1$ [22].

Advances in genetic and sequencing have identified a plethora of disease-associated and disease-causing genetic alterations [23]. In the recent study, PLOD2 was reported to serve as a prognostic marker in laryngeal cancer by promoting cancer stem cell-like characteristics via Wnt signaling pathway [24]. However, there are no relevant studies completely elucidated whether PLOD2 effect on CSCs confers drug resistance in laryngeal cancers. Accordingly, the aim of our study is to investigate the potential effects of PLOD2 against LC and to explore the possible role of PLOD2 and Integrin $\beta 1$ on drug resistance in LC, as possible protective mechanisms.

Materials and methods

Cell lines and cell culture

Tu686 and TU138 Squamous cell carcinoma of head and neck (SCCHN) cell strains (Tiancheng Technology, Shanghai, China) were cultured in the Roswell Park Memorial Institute (RPMI) 1640 culture medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were maintained in 5% CO₂ with 95% air incubator at 37 °C. Cisplatin (DDP)-resistance (R) laryngeal squamous carcinoma cells (TU686-DDP-R, TU138-DDP-R) came from TU686 and TU138 cells with continuous cisplatin treatment.

Cell viability

The cell survival rate of cells was analyzed by cell-counting kit-8 (CCK-8) method according to the manufacturer's instructions. Briefly, Tu686 and TU138 cells in the logarithmic growth phase were seeded into 96-well plates and cultured overnight at 37 °C, 5% CO₂. Each hole was added with 10 μ L CCK-8 solution (Beyotime, Shanghai, China). After incubating for 2 h, the optical density (OD) at 450 nm was measured by Elx800 Reader (Bio-Tek Instruments Inc., Winooski, VT, USA).

siRNA and plasmid transfection

The specific small interfering RNA that targeted PLOD2 (siRNA-PLOD2) and a scrambled negative control (siRNA-NC) came from Gene-Pharma (Shanghai, China). The expression vector expressing PLOD2 (pcDNA3.1-PLOD2) and the negative control were also constructed and purchased from Gene-Pharma (Shanghai, China). To augment the expression of PLOD2, Tu686 and TU138 cell lines were cultured in 60 mm dishes overnight. Then, pcDNA3.1-PLOD2 vector or siRNA was infected into Tu686 and TU138 cells for 48 h at 37 °C. Subsequently, the cell medium supernatant was collected to obtain adenovirus-PLOD2 (Ad-PLOD2), which was used to transfect into Tu686 and TU138 cell lines for 48 h at 37 °C using Opti-MEM medium and Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Transwell

Matrigel was taken out from -20 °C and melted at 4 °C. Then Matrigel was diluted to a final concentration of 300 μ L/mL with serum-

free cell culture medium. Cell invasion was examined using a 24-well Transwell insert system contained 800 μ L of 10% FBS DMEM medium. 100 μ L of Matrigel was added to the upper chamber of the transwell and incubated at 37 °C for 3 h to form a gel. After Matrigel was gelatinized, a 4×10^4 cell suspension was inserted into the transwell upper chamber. Meanwhile, 600–800 μ L medium containing 10% serum to the lower chamber and the system was cultured at 37 °C in a 5% CO₂ incubator for 48 h. Cells were cultured in an incubator for 48 h, and the chamber was carefully rinsed with PBS. The cells were soaked in 70% methanol solution for 1 h, stained with 0.5% crystal violet stain, placed at room temperature for 20 min, and the unmigrated cells on the upper chamber side were wiped clean with a sterile cotton ball. The invasive cells on the below side of chamber were collected and examined under microscope.

Flow cytometry for cell apoptosis

The apoptotic cells were measured by Annexin V- fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (key-gentec, Nanjing, China) according to the manufacturer's instructions. The cells in the logarithmic growth phase were digested and inoculated into a six-well plate, digested and rinsed twice with PBS buffer solution. Then, Annexin V- FITC and PI were added to each sample and cultured at room temperature for 5–15 min in the dark and analyzed using FACS flow cytometry (BD, San Jose, CA, USA).

Detection of CD44 and CD133 with immunofluorescence

CD44 and CD133 activities were measured by CD44 or CD133 Deacetylase Fluorometric Assay kit (BioVision Inc, Milpitas Blvd, Calif, USA) according to the manufacturer's instructions.

Xenograft model

BALB/c nude mice were purchased from Jinan Pengyue Laboratory Animal Breeding Co., Ltd. Cells in different groups were subcutaneously injected into the left axilla of nude mice at a dose of 1×10^7 . After tumor formation, the tumors were measured with an electronic vernier caliper and the tumor volume was calculated. The tumors were further observed for 30 days, and the tumor growth rate was calculated. All the experiment procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996). Our animal studies were approved by Yantaishan hospital and received humane care.

TUNEL staining

TUNEL fluorescent assay was con according to the instructions of the ducted with an *in-situ* Cell Death Detection Kit (Roche Inc., Indianapolis, IN, United States). Fluorescence microscopy was employed to observe apoptotic cells and the images were photographed at 400 \times magnification.

Western blotting

Protein concentrations of tumors or the cells proteins (20 μ g) were separated by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following by the standard protocol as previous described, the proteins were transferred into a nitrocellulose (NC) membrane which was rinsed and blocked with 5% skim milk for 1 h at room temperature. Then, the following primary antibodies: PLOD2 (1:500, LSBio, Seattle, USA), Integrin $\beta 1$ (1:500, abcam, Cambridge, UK), MRP1 (1:500, abcam, Cambridge, UK), P-gp (1:1000; abcam, Cambridge, UK) were added incubated overnight at 4 °C. After washing, the complexes were incubated with a horse radish peroxidase (HRP)-coupled secondary antibody (1:1000, abcam, Cambridge, UK) at 37 °C for 1.5 h, then visualized with an enhanced chemiluminescence system.

The density of each protein band was analyzed using Image-ProPlus6.0 (Media Cybernetics, Silver Spring, U.S.A.).

Statistical analysis

All experimental data were analyzed using IBM SPSS Statistics Version 21.0 (SPSS Inc., Chicago, IL, USA). The significant difference of

data in for comparison of three or more groups was analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's post-test, a value of $p < 0.05$ was statistically significant.

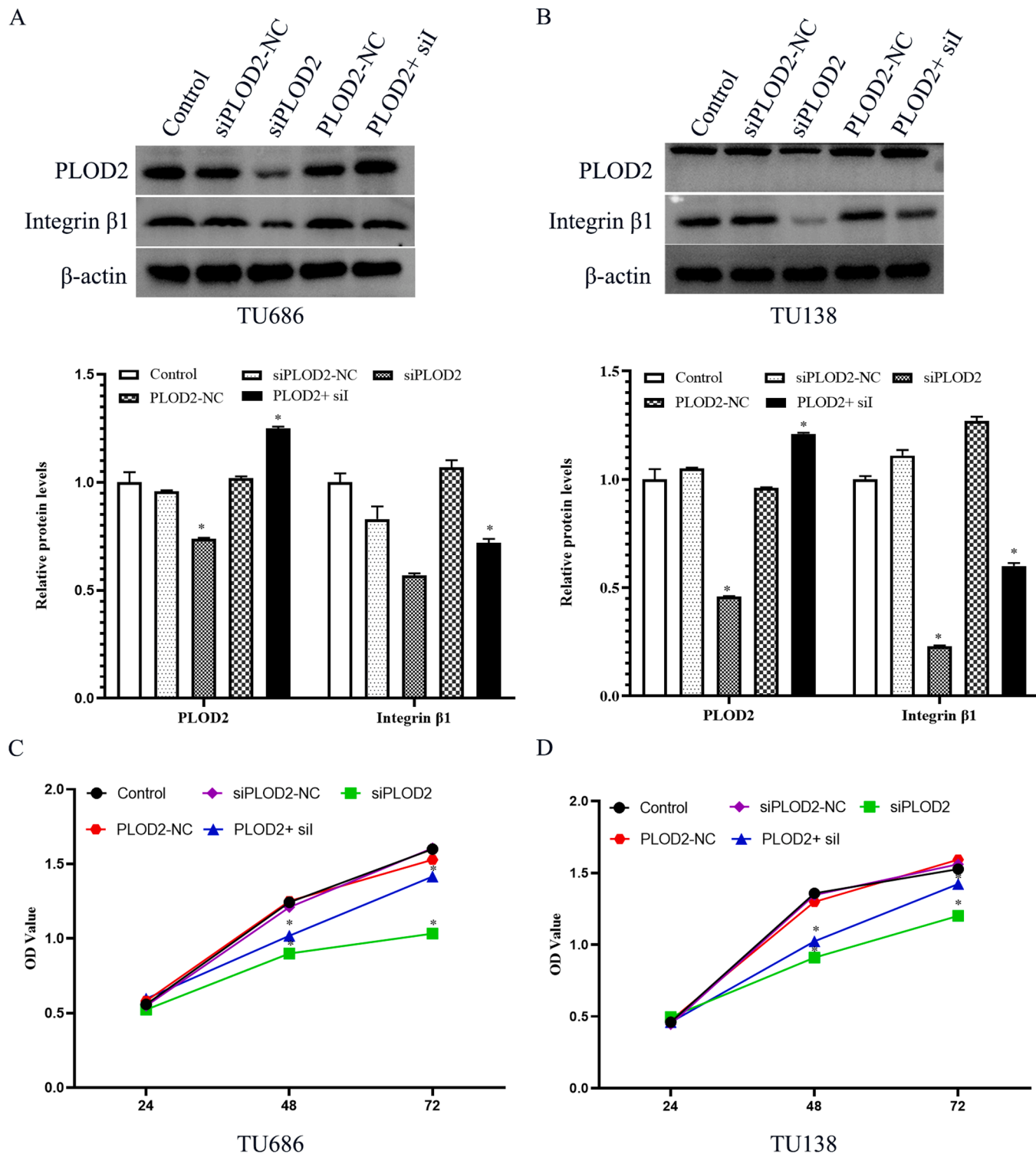


Fig. 1. Effects of PLOD2 on relative protein levels and cell viability in Tu686 and TU138 cells. A: PLOD2 and Integrin β1 protein levels in Tu686 cells were detected by Western blot assay; B: PLOD2 and Integrin β1 protein levels in TU138 cells were detected by Western blot assay; C: Tu686 cell viability was detected by CCK-8 assay; D: TU138 cell was detected by CCK-8 assay. * $p < 0.05$ vs. Control group.

Results

Downregulation of PLOD2 attenuated the Integrin $\beta 1$ expression in Tu686 and TU138 cells

To detect the effect of PLOD2 on Tu686 and TU138 cell viability, the cells were divided in five groups: the Control group, siPLOD2-NCgroup, siPLOD2 group, PLOD2-NC group and PLOD2- siIntegrin $\beta 1$ group.

Firstly, to determine the expression of PLOD2 expression in Tu686 and TU138 cells, we performed western blot analyses. As shown in Fig. 1A and B, compared with Control group, siPLOD2 group express relatively lower expression of PLOD2 while PLOD2-mimic group showed higher expression of PLOD2 both in Tu686 and TU138 cells ($p < 0.05$). Interestingly, siPLOD2 induced the decrease of Integrin $\beta 1$, thus Integrin $\beta 1$ expression is consistent with the expression of PLOD2. Meanwhile, the increase of Integrin $\beta 1$ in cells caused by the transfection of PLOD2 mimic was effectively decreased by si Integrin $\beta 1$ ($p < 0.05$). No differences were found in the Control group, siPLOD2-NC group, and PLOD2-NC group.

Downregulation of PLOD2 attenuated the cell proliferation, invasion and apoptosis in Tu686 and TU138 cells

Then, in order to determine whether PLOD2 affected cell proliferation and invasion in Tu686 and TU138 cells, CCK-8 and Transwell assays were performed. The growth curves obtained from CCK-8 assay showed that SiPLOD2 significantly repressed the ability of cell proliferation in Tu686 and TU138 cells ($p < 0.05$, vs Control group). Moreover, the results of Transwell assay confirmed that Tu686 and TU138 cells invasion were severely suppressed by SiPLOD2 ($p < 0.05$, vs Control group). Accordingly, the increase of cell proliferation and invasion of Tu686 and TU138 cells caused by the transfection of PLOD2 mimic were effectively rescued by si Integrin $\beta 1$ ($p < 0.05$, vs Control group). Meanwhile, no differences were found in the Control group, siPLOD2-NCgroup, and PLOD2-NC group.

Additionally, Flow cytometry was used to detect the apoptotic rate of Tu686 and TU138 cells. As shown in Fig. 2, SiPLOD2 significantly increased the cell apoptosis in Tu686 and TU138 cells ($p < 0.05$, vs Control group). Meanwhile, the decrease of cell apoptosis in Tu686 and TU138 cells induced by PLOD2 mimic was effectively rescued by si Integrin $\beta 1$ ($p < 0.05$, vs Control group). Meanwhile, no differences were found in the Control group, siPLOD2-NCgroup, and PLOD2-NC group.

Downregulation of PLOD2 attenuated the CSCs properties in Tu686 and TU138 cells

Then, in order to detect the relationship between PLOD2 and Integrin $\beta 1$ in CSCs properties, CD44 and CD133 were detected to identify CSCs. As shown in Fig. 3, SiPLOD2 significantly repressed CD44 and CD133 expression in Tu686 and TU138 cells ($p < 0.05$, vs Control group). Accordingly, the levels of CD44 and CD133 expression in Tu686 and TU138 cells were significantly increased by the transfection of PLOD2 mimic ($p < 0.05$, vs Control group). Meanwhile, no differences were found in the Control group, siPLOD2-NCgroup, and PLOD2-NC group.

Downregulation of PLOD2 attenuated the tumor growth and apoptosis in vivo

To further evaluate the effect of PLOD2 silencing on tumor growth, cells were subcutaneously injected into the left axilla of nude mice to set xenograft model. We added PLOD2 mimic+Integrin $\beta 1$ si-RNA group to detect the relationship between PLOD2 and Integrin $\beta 1$ in CSCs properties. As shown in Fig. 4, the tumor growth curve indicated that the tumor volume in the SiPLOD2 group at 18, 24, 30 days point was smaller ($p < 0.05$, vs Control group). Meanwhile, the increase of tumor volume

caused by the transfection of PLOD2 mimic was effectively rescued by si Integrin $\beta 1$ ($p < 0.05$, vs Control group).

Apoptotic cells in the tumors were detected through TUNEL assay. As shown in Fig. 5A, SiPLOD2 significantly increased the cell apoptosis in tumors ($p < 0.05$, vs Control group). Interestingly, the decrease of cell apoptosis in tumors caused by the transfection of PLOD2 mimic was effectively rescued by si Integrin $\beta 1$. Meanwhile, no differences were found in the Control group, siPLOD2-NCgroup, and PLOD2-NC group.

Downregulation of PLOD2 attenuated the CSCs properties in vivo

Then, in order to detect the relationship between PLOD2 and Integrin $\beta 1$ in CSCs properties *in vivo*, CD44 and CD133 in xenograft model were detected to identify CSCs. As shown in Fig. 5B and C, SiPLOD2 significantly repressed CD44 and CD133 expression in xenograft mice ($p < 0.05$, vs Control group). Meanwhile, the increase of CD44 and CD133 expression in xenograft mice caused by the transfection of PLOD2 mimic was effectively rescued by si Integrin $\beta 1$ ($p < 0.05$, vs Control group). Meanwhile, no differences were found in the Control group, siPLOD2-NCgroup, and PLOD2-NC group.

Downregulation of PLOD2 attenuated the cell proliferation, invasion and apoptosis in cisplatin-resistant Tu686 and TU138 cells

To detect the relationship between PLOD2 and Integrin $\beta 1$ in CSCs properties in cisplatin-resistant cells, the cisplatin-resistant cells were divided in four groups: the control group, siPLOD2 -DDP-R group, PLOD2- DDP-R group, PLOD2+ Integrin $\beta 1$ - DDP-R group. In order to determine whether PLOD2 affected apoptosis in cisplatin-resistant Tu686 and TU138 cells, Flow cytometry assay was performed.

As shown in Fig. 6A and B, SiPLOD2 significantly increased the cell apoptosis in cisplatin-resistant Tu686 and TU138 cells ($p < 0.05$, vs Control group). Accordingly, cell apoptosis in isplatin-resistant Tu686 and TU138 cells was significantly decreased by the transfection of PLOD2 mimic ($p < 0.05$, vs Control group). Interestingly, the decrease of cell apoptosis in cisplatin-resistant Tu686 and TU138 cells caused by the transfection of PLOD2 mimic was effectively rescued by si Integrin $\beta 1$ ($p < 0.05$, vs PLOD2 group).

Downregulation of PLOD2 attenuated the CSCs properties in cisplatin-resistant Tu686 and TU138 cells

Then, in order to detect the relationship between PLOD2 and Integrin $\beta 1$ in CSCs properties, CD44 and CD133 were detected to identify CSCs. As shown in Fig. 6C and D, SiPLOD2 significantly repressed CD44 and CD133 expression in cisplatin-resistant Tu686 and TU138 cells ($p < 0.05$, vs Control group). Accordingly, the levels of CD44 and CD133 expression in cisplatin-resistant Tu686 and TU138 cells were significantly increased by the transfection of PLOD2 mimic ($p < 0.05$, vs Control group). Meanwhile, the increase of CD44 and CD133 expression in cisplatin-resistant Tu686 and TU138 cells caused by the transfection of PLOD2 mimic was effectively rescued by si Integrin $\beta 1$ ($p < 0.05$, vs PLOD2 group).

Downregulation of PLOD2 attenuated the MRP1 and P-gp expression in cisplatin-resistant Tu686 and TU138 cells

To determine the expression of MRP1 and P-gp in cisplatin-resistant Tu686 and TU138 cells, we performed western blot analyses. As shown in Fig. 7A and B, compared with Control group, SiPLOD2 significantly repressed MRP1 and P-gp expression in cisplatin-resistant Tu686 and TU138 cells ($p < 0.05$, vs Control group). Accordingly, the levels of MRP1 and P-gp expression in cisplatin-resistant Tu686 and TU138 cells were significantly increased by the transfection of PLOD2 mimic ($p < 0.05$, vs Control group). Meanwhile, the increase of MRP1 and P-gp expression in cisplatin-resistant Tu686 and TU138 cells caused by the

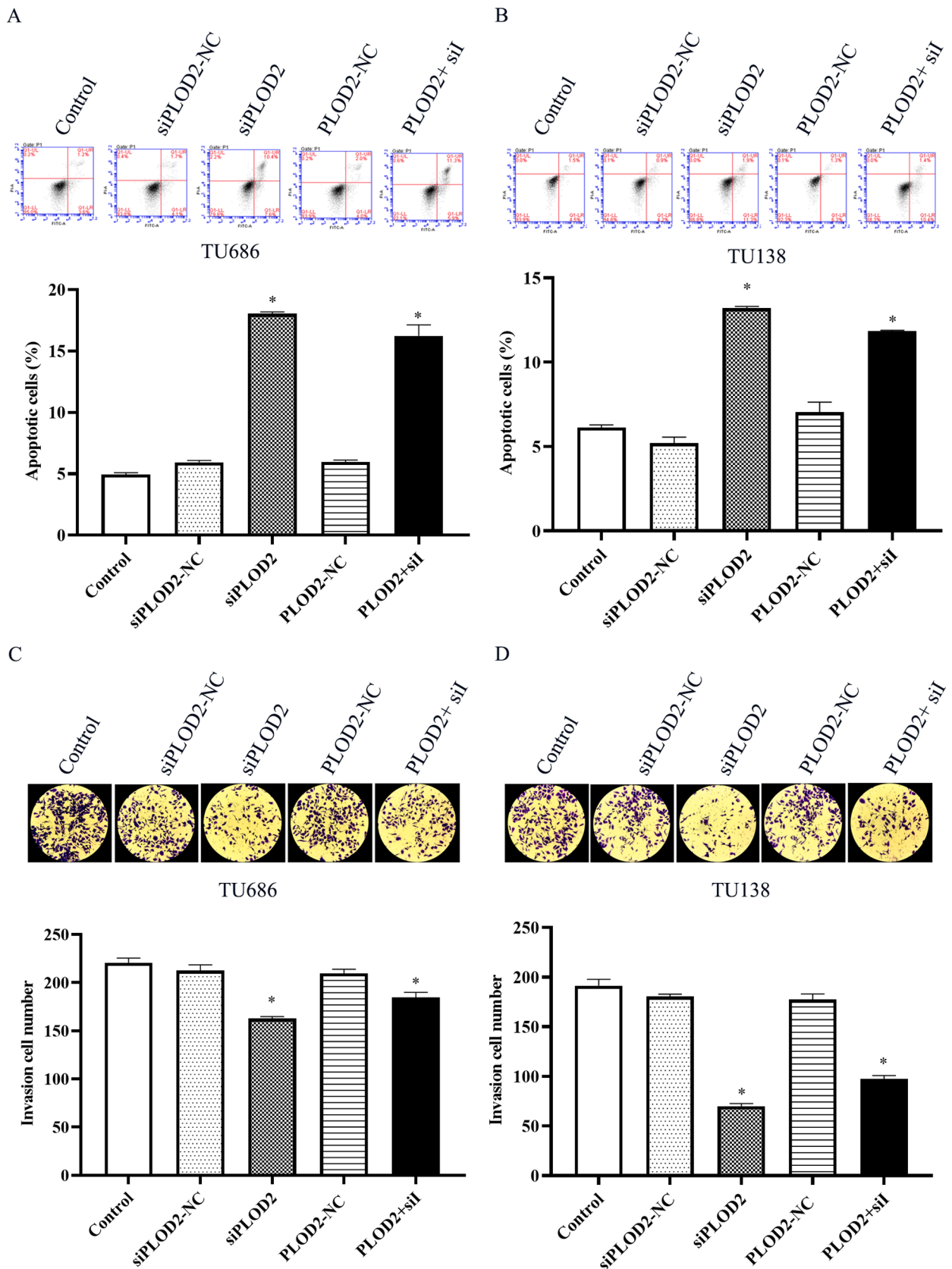


Fig. 2. Effects of PLOD2 on cell apoptosis and cell invasion in Tu686 and TU138 cells. A: Cell apoptosis in Tu686 cells were detected by Flow cytometry. B: Cell apoptosis in TU138 cells were detected by Flow cytometry. C: Cell invasion in Tu686 cells were detected by Transwell. D: Cell invasion in TU138 cells were detected by Transwell. * $p < 0.05$ vs. Control group.

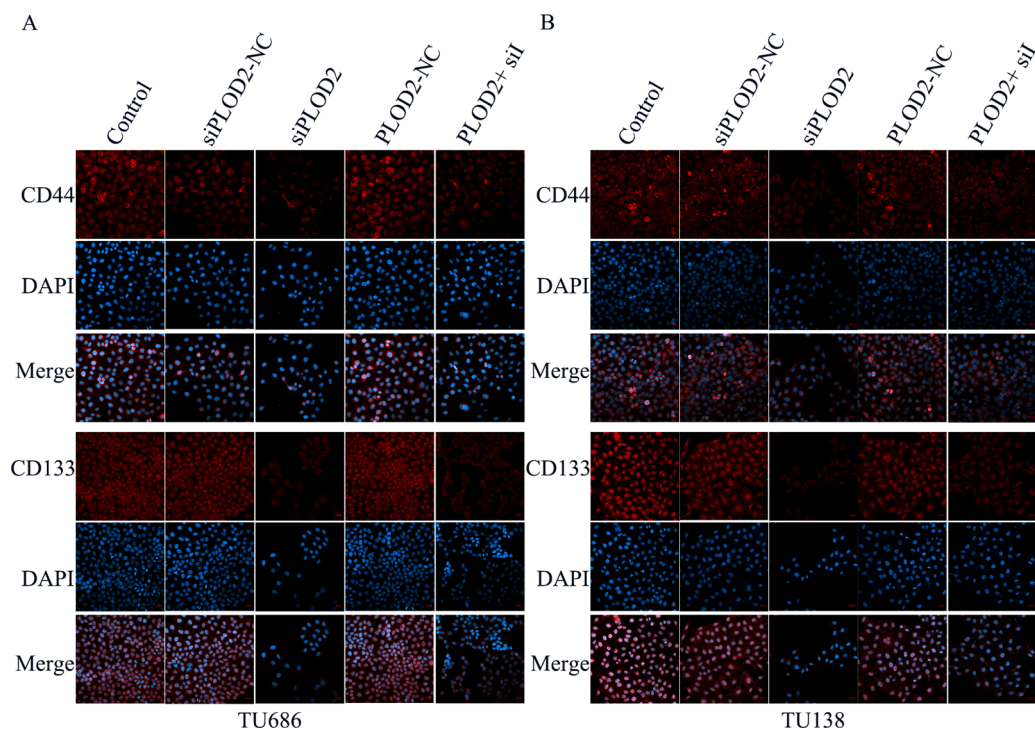


Fig. 3. Effects of PLOD2 on CD44 and CD133 expressions in Tu686 and TU138 cells. A: CD44 and CD133 expression in Tu686 cells; B: CD44 and CD133 expression in TU138 cells. * $p < 0.05$ vs. Control group.

transfection of PLOD2 mimic was effectively rescued by si Integrin $\beta 1$ ($p < 0.05$, vs PLOD2 group).

Downregulation of PLOD2 attenuated the tumor growth and apoptosis in vivo

To further evaluate the effect of PLOD2 silencing on tumor growth, a cisplatin-resistant Tu686 and TU138 cells xenograft model was constructed. As shown in Fig. 7C and D, the tumor growth curve indicated that the tumor volume in the SiPLOD2 group at each time point was smaller ($p < 0.05$, vs Control group). Accordingly, the tumor volume in cisplatin-resistant Tu686 and TU138 cells xenograft model was significantly increased by the transfection of PLOD2 mimic ($p < 0.05$, vs Control group). Meanwhile, the increase of tumor volume caused by the transfection of PLOD2 mimic was effectively rescued by si Integrin $\beta 1$ ($p < 0.05$, vs PLOD2 group).

Apoptotic cells in the tumors were detected through TUNEL assay. As shown in Fig. 8A, SiPLOD2 significantly increased the cell apoptosis in tumors ($p < 0.05$, vs Control group). Accordingly, the tumor volume in cisplatin-resistant tumors were significantly increased by the transfection of PLOD2 mimic ($p < 0.05$, vs Control group). Interestingly, the decrease of cell apoptosis in tumors caused by the transfection of PLOD2 mimic was effectively rescued by si Integrin $\beta 1$ ($p < 0.05$, vs PLOD2 group).

Downregulation of PLOD2 attenuated the CSCs properties in vivo

Then, in order to detect the relationship between PLOD2 and Integrin $\beta 1$ in CSCs properties in cisplatin-resistant tumors, CD44 and CD133 in xenograft model were detected to identify CSCs. As shown in Fig. 8B and C, SiPLOD2 significantly repressed CD44 and CD133 expression ($p < 0.05$, vs Control group). Accordingly, CD44 and CD133 expressions were significantly increased by the transfection of PLOD2 mimic ($p < 0.05$, vs Control group). Meanwhile, the increase of CD44 and CD133 expression caused by the transfection of PLOD2 mimic was effectively rescued by si Integrin $\beta 1$ ($p < 0.05$, vs PLOD2 group).

Discussion

Laryngeal cancer is an especially aggressive and predominantly malignant tumor of HNSCCs. Despite advances in the diagnosis and effective clinical treatment, the mortality rate is still having not been effectively reduced and prognosis of LC is still poor [25]. Delineating disease-associated genetic variation is the focus of intensive research, a detailed understanding of the restructuring of the proteomics signaling cascades PLOD2 on LC will provide new avenues to explore its use for related malignancies [26,27]. Therefore, the mechanism underlying LC resistance and novel therapeutic approaches for the eradication of LC needs to be investigated.

Studies show that CSCs are characterized by the self-renewal, invasiveness and metastasis capacity in LC [28]. To investigate the effect of PLOD2 on LC stem cell-like features, we studied the cell viability, invasion and apoptotic rate of Tu686 and TU138 cells. These findings suggest that overexpression of PLOD2 could increase Tu686 and TU138 cells stem cell like characteristics, including their viability, invasion rate and apoptotic rate. For the identification of CSCs, cell surface biomarkers such as CD44, CD117, and CD133 can be used as the markers of stemness [28]. CD133 and CD44 are specific markers of CSCs stemness and their expressions strongly predicts poorer prognosis and survival rate in oral cancer cell line [29]. Previous study revealed that CD44+/CD133+/CD117+ cells obtained from a Hep2 cell line showed higher migration capacity [11]. In this study, CD44 and CD133 are used as markers to identify the CSCs in cancers. Results showed that the levels of stem cell markers CD44 and CD133 were enhanced in Tu686 and TU138 cells. On the contrary, the silencing of PLOD2 could inhibit LC cells stemness and decreased the levels of CD44 and CD133.

Previous studies found that PLOD2 was a key regulator of LC invasion/metastasis through its specific interaction with integrin $\beta 1$. In our study, LC tumorigenesis in nude mice were induced. Our research results suggest that siPLOD2 could decrease the tumor weight and volume whereas increase the apoptotic rate and stem cell markers expression levels in tumor, consistent with the results *in vitro*. On the contrary, overexpression of PLOD2 could increase the cells stem cell like

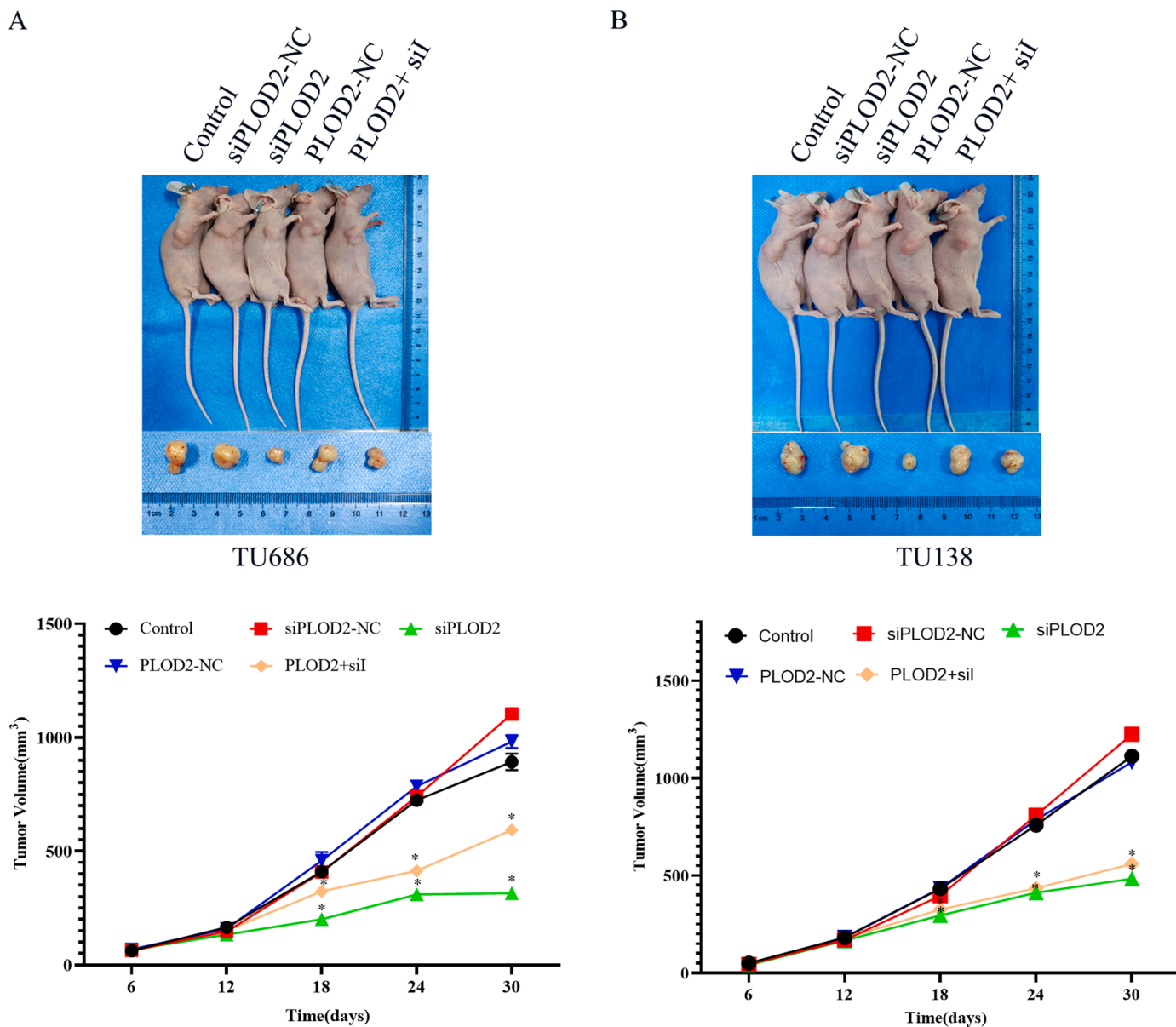


Fig. 4. Effects of PLOD2 on tumor volume in xenograft model. A: Tumor volume of TU686 cells induced xenograft model; B: Tumor volume of TU138 cells induced xenograft model. * $P < 0.05$ vs. Sham group, # $P < 0.05$ vs. MI/R group, & $P < 0.05$ vs. MI/R+Ator group. * $p < 0.05$ vs. Control group.

characteristics and the levels of CD44 and CD133. Interestingly, when the si Integrin $\beta 1$ added, the effects caused by PLOD2 were eliminated. Taken together, PLOD2 could markedly regulate LC cells stemness and might play a pivotal role for increased tumorigenesis in nude mice via Integrin $\beta 1$.

Additionally, an increasing number of reports suggested CSCs play a pivotal role in drug assistance, but the relationship between PLOD2 and Integrin $\beta 1$ in Cisplatin (DDP)-resistance (R) laryngeal squamous carcinoma still remains unknown [30]. Then we detect the effect of PLOD2 on DDP-R LC cells and tumorigenesis in nude mice with cisplatin-resistant LC. Based on results of our analysis, PLOD2 could markedly regulate DDP-R LC cells stemness and tumorigenesis in cisplatin-resistant nude mice via Integrin $\beta 1$.

In clinical treatment, LC patients are mainly treated with laryngeal surgery followed by radiation/chemotherapy depending on the disease stage [31,32]. Effectiveness of chemotherapy is thwarted by drug accumulation induced multidrug resistance. MRP1 is a member of MRP transporter family, its increased expression is often detected in various types of cancer [33]. P-glycoprotein (P-gp) acts as a promiscuous drug

efflux pump in the cell membrane and is overexpression in cancer cells. Suppressing the expression of P-gp has been recognized as a viable target to overcome multidrug resistance in cancer [34]. Accumulating evidence has showed that P-gp and MRP1 are two key transporters found to be involved in mediate drug resistance [35]. Western blot analyses of P-gp and MRP1 were taken to support our results. SiPLOD2 significantly repressed MRP1 and P-gp expression in xenograft mice via Integrin $\beta 1$. Our results were consistent with the previous studies [36].

Conclusion

In summary, suppressing the expression of PLOD2 could meditate LC stem cell-like features by decrease cell viability and invasion and increase apoptotic rate via Integrin $\beta 1$. Meanwhile, the inhibition of PLOD2 expression could decrease P-gp and MRP1 expression thus markedly regulate DDP-R LC cells stemness and tumorigenesis via Integrin $\beta 1$.

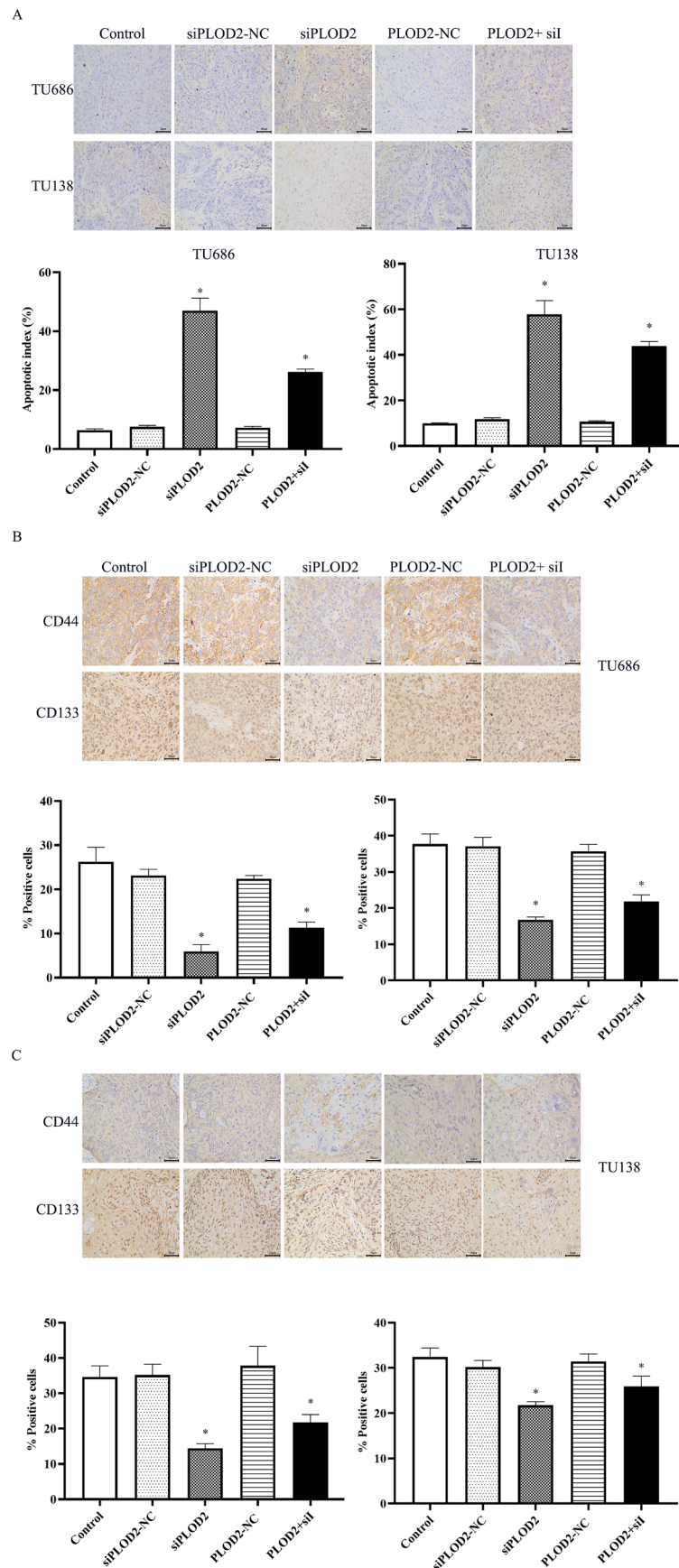


Fig. 5. Effects of PLOD2 on tumor apoptosis and CD44 and CD133 expressions in xenograft model. A: Tumor apoptosis of TU686 cells induced xenograft model was detected by TUNEL assay; B: Tumor apoptosis of TU138 cells induced xenograft model was detected by TUNEL assay; C: CD44 and CD133 expressions in Tu686 cells induced xenograft model; D: CD44 and CD133 expression in TU138 cells induced xenograft model. * $p < 0.05$ vs. Control group.

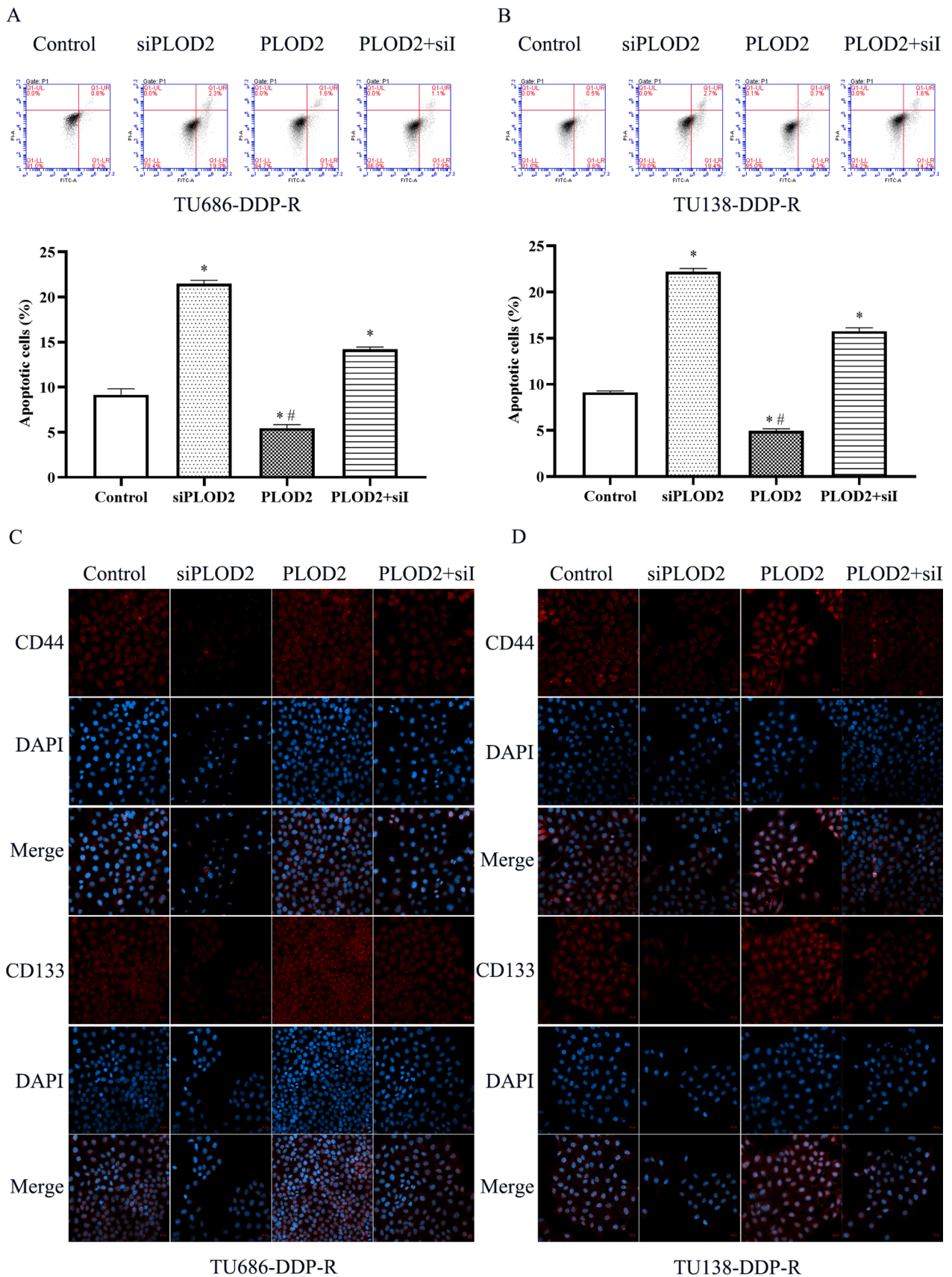


Fig. 6. Effects of PLOD2 on cell apoptosis, CD44 and CD133 expressions in Tu686-DDP-R and TU138-DDP-R cells. A: Cell apoptosis in Tu686-DDP-R cells were detected by Flow cytometry. B: Cell apoptosis in TU138-DDP-R cells were detected by Flow cytometry. C: CD44 and CD133 expression in Tu686-DDP-R cells; D: CD44 and CD133 expression in TU138-DDP-R cells. * $p < 0.05$ vs. Control group, # $p < 0.05$ vs. PLOD2 group.

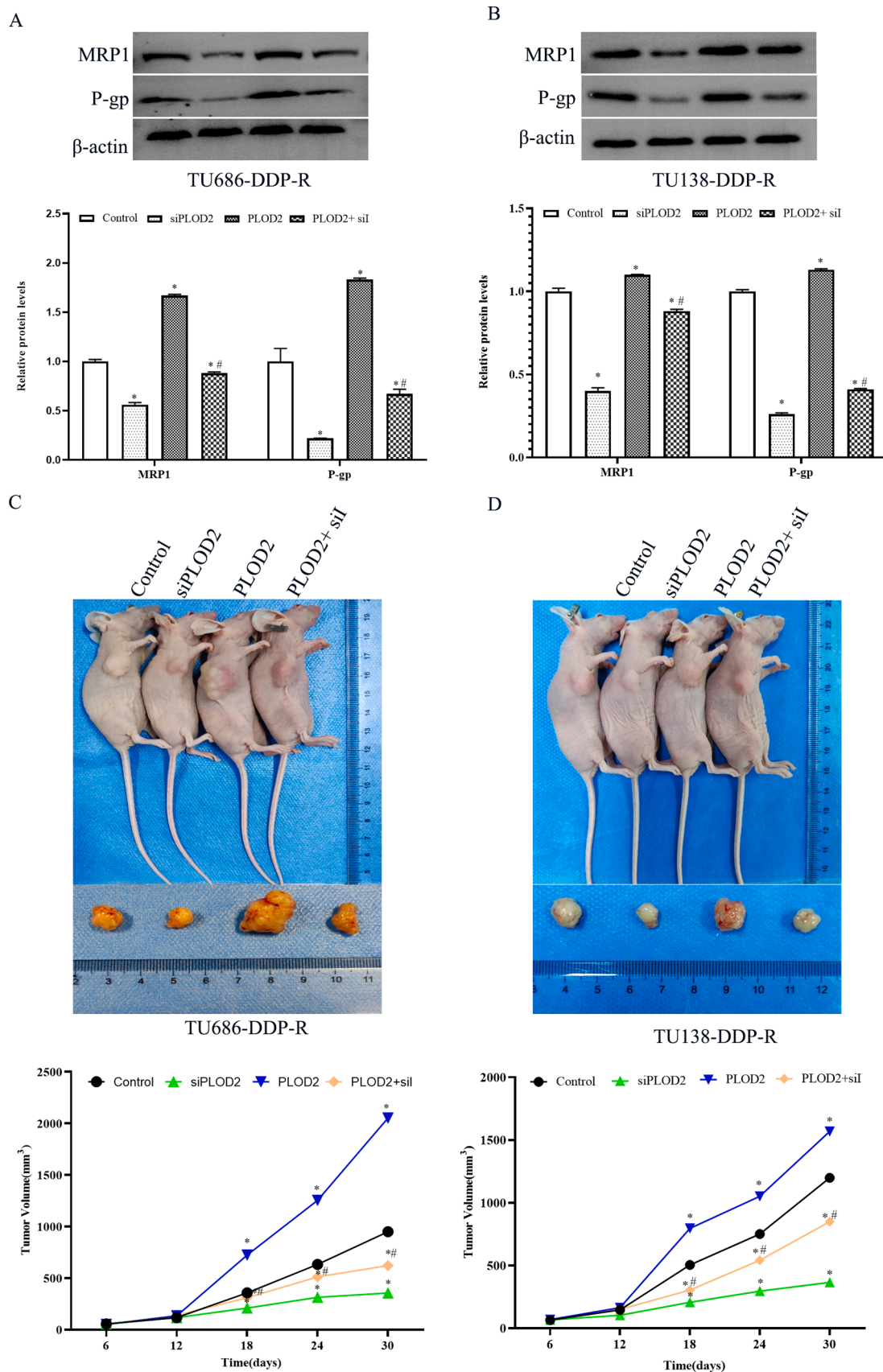


Fig. 7. Effects of PLOD2 on relative protein levels and tumor volume in xenograft model. A: MRP1 and P-gp levels in Tu686-DDP-R cells were detected by Western blot. B: MRP1 and P-gp levels in Tu138-DDP-R cells were detected by Western blot. C: Tumor volume of Tu686-DDP-R cells induced xenograft model; D: Tumor volume of Tu138-DDP-R cells induced xenograft model. * $p < 0.05$ vs. Control group, # $p < 0.05$ vs. PLOD2 group.

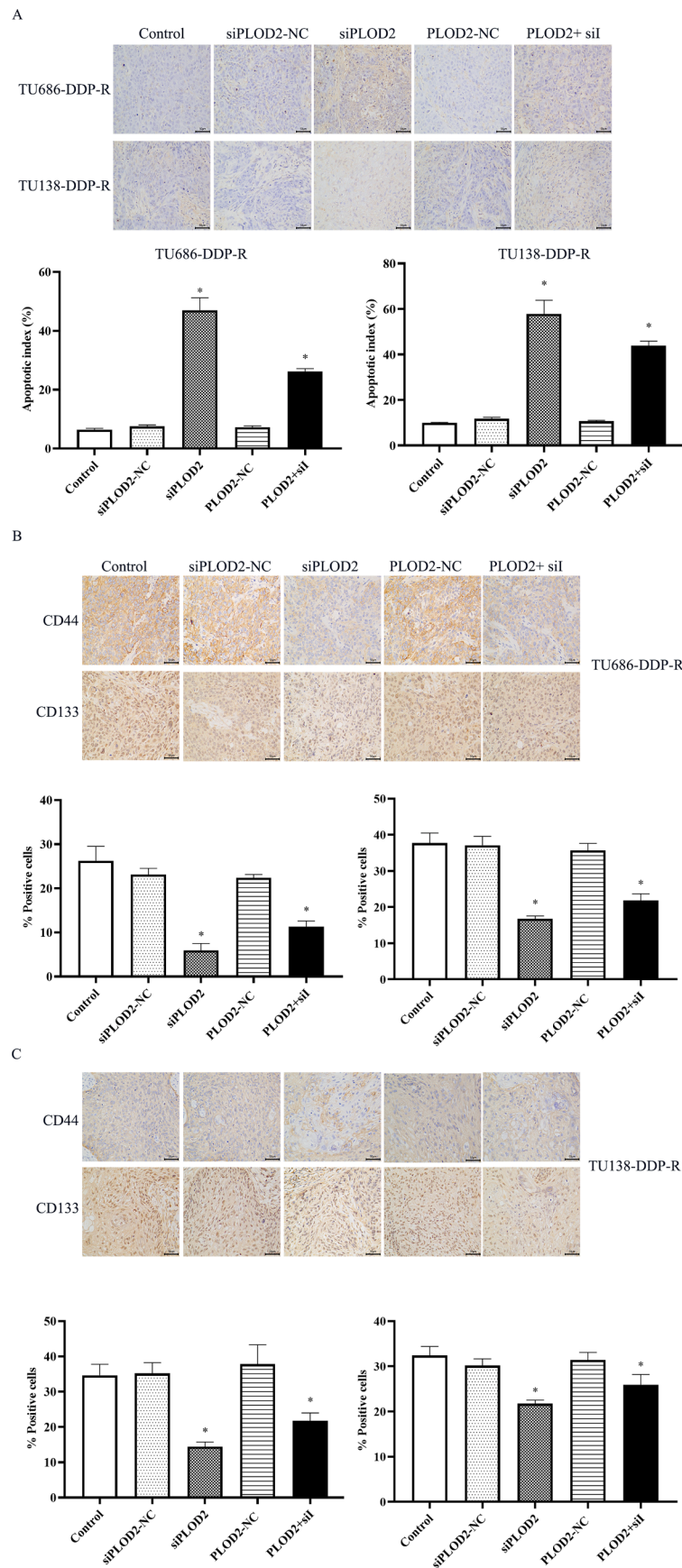


Fig. 8. Effects of PLOD2 on tumor apoptosis and CD44 and CD133 expressions in xenograft model. A: Tumor apoptosis of Tu686-DDP-R cells induced xenograft model was detected by TUNEL assay; B: Tumor apoptosis of Tu138-DDP-R cells induced xenograft model was detected by TUNEL assay; C: CD44 and CD133 expressions in Tu686-DDP-R cells induced xenograft model; D: CD44 and CD133 expression in Tu138-DDP-R cells induced xenograft model.

Data availability

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

CRedit authorship contribution statement

Meiyan Song: Visualization, Methodology, Supervision, Writing – review & editing. **Xing Liu:** Visualization, Methodology, Supervision, Writing – review & editing. **Tao Li:** Data curation. **Yueqin Zhang:** Formal analysis. **Xiaoyan Zhao:** Methodology. **Wen Sun:** Methodology. **Zhen Li:** Writing – original draft.

Declaration of Competing Interest

The authors declared that there were no conflicts of interest in the study.

Acknowledgment

Not applicable.

References

- [1] D.E. Johnson, B. Burtneess, C.R. Leemans, V.W.Y. Lui, J.E. Bauman, J.R. Grandis, Head and neck squamous cell carcinoma, *Nat. Rev. Dis. Prim.* 6 (1) (2020) 92.
- [2] J.D. McDermott, D.W. Bowles, Epidemiology of head and neck squamous cell carcinomas: impact on staging and prevention strategies, *Curr. Treat. Options Oncol.* 20 (5) (2019) 43.
- [3] R.V. Smith, Transoral robotic surgery for larynx cancer, *Otolaryngol. Clin. N. Am.* 47 (3) (2014) 379–395.
- [4] C. Saraniti, R. Speciale, M. Santangelo, N. Massaro, A. Maniaci, S. Gallina, et al., Functional outcomes after supracricoid modified partial laryngectomy, *J. Biol. Regul. Homeost. Agents* 33 (6) (2019) 1903–1907.
- [5] G. Meccariello, A. Maniaci, G. Bianchi, G. Cammaroto, G. Iannella, A. Catalano, et al., Neck dissection and trans oral robotic surgery for oropharyngeal squamous cell carcinoma, *Auris Nasus Larynx* 49 (1) (2022) 117–125.
- [6] F. Li, J. Xu, S. Liu, Cancer stem cells and neovascularization, *Cells* 10 (5) (2021) 1070.
- [7] C. Sun, X. Dai, D. Zhao, H. Wang, X. Rong, Q. Huang, et al., Mesenchymal stem cells promote glioma neovascularization *in vivo* by fusing with cancer stem cells, *BMC Cancer* 19 (1) (2019) 1240.
- [8] A. Greco, M.I. Rizzo, A. De Virgilio, A. Gallo, M. Fusconi, G. Pagliuca, et al., Cancer stem cells in laryngeal cancer: what we know, *Eur. Arch. Otorhinolaryngol.* 273 (11) (2016) 3487–3495.
- [9] S. Lu, J. Tian, Z. Lv, H. Wang, X. Bai, W. Liu, et al., The probable role of tumor stem cells for lymph node metastasis in supraglottic carcinoma, *Pathol Oncol Res* 17 (1) (2011) 33–38.
- [10] M. Boxberg, C. Gotz, S. Haidari, C. Dorfner, M. Jesinghaus, E. Drecoll, et al., Immunohistochemical expression of CD44 in oral squamous cell carcinoma in relation to histomorphological parameters and clinicopathological factors, *Histopathology* 73 (4) (2018) 559–572.
- [11] A.L. Silva Galbiatti-Dias, G.M.M. Fernandes, M.M.U. Castanhole-Nunes, L. F. Hidalgo, C.H.V. Nascimento Filho, R.S. Kawasaki-Oyama, et al., Relationship between CD44(high)/CD133(high)/CD117(high) cancer stem cells phenotype and cetuximab and paclitaxel treatment response in head and neck cancer cell lines, *Am. J. Cancer Res.* 8 (8) (2018) 1633–1641.
- [12] G.M.M. Fernandes, A.L.S. Galbiatti-Dias, L.A.M. Ferreira, V. Serafim Junior, G. H. Rodrigues-Fleming, J.G. de Oliveira-Cucolo, et al., Anti-EGFR treatment effects on laryngeal cancer stem cells, *Am. J. Transl. Res.* 13 (1) (2021) 143–155.
- [13] H. Du, Y. Chen, X. Hou, Y. Huang, X. Wei, X. Yu, et al., PLOD2 regulated by transcription factor FOXA1 promotes metastasis in NSCLC, *Cell Death Dis.* 8 (10) (2017) e3143.
- [14] H. Du, M. Pang, X. Hou, S. Yuan, L. Sun, PLOD2 in cancer research, *Biomed. Pharmacother.* 90 (2017) 670–676.
- [15] D. Barkan, A.F. Chambers, beta1-Integrin: a potential therapeutic target in the battle against cancer recurrence, *Clin. Cancer Res.* 17 (23) (2011) 7219–7223.
- [16] Y. Sun, S. Wang, X. Zhang, Z. Wu, Z. Li, Z. Ding, et al., Identification and validation of PLOD2 as an adverse prognostic biomarker for oral squamous cell carcinoma, *Biomolecules* 11 (12) (2021) 1842.
- [17] S. Cheriyaundath, A. Kumar, N. Gavert, T. Brabletz, A. Ben-Ze'ev, The collagen-modifying enzyme PLOD2 is induced and required during L1-mediated colon cancer progression, *Int. J. Mol. Sci.* 22 (7) (2021) 3552.
- [18] X. Wang, J. Guo, M. Dai, T. Wang, T. Yang, X. Xiao, et al., PLOD2 increases resistance of gastric cancer cells to 5-fluorouracil by upregulating BCRP and inhibiting apoptosis, *J. Cancer* 11 (12) (2020) 3467–3475.
- [19] W. Du, N. Liu, Y. Zhang, X. Liu, Y. Yang, W. Chen, et al., PLOD2 promotes aerobic glycolysis and cell progression in colorectal cancer by upregulating HK2, *Biochem. Cell Biol.* 98 (3) (2020) 386–395.
- [20] C. Margadant, H.N. Monsuur, J.C. Norman, A. Sonnenberg, Mechanisms of Integrin activation and trafficking, *Curr. Opin. Cell Biol.* 23 (5) (2011) 607–614.
- [21] Y. Ueki, K. Saito, H. Iioaka, I. Sakamoto, Y. Kanda, M. Sakaguchi, et al., PLOD2 is essential to functional activation of Integrin beta1 for invasion/metastasis in head and neck squamous cell carcinomas, *iScience* 23 (2) (2020), 100850.
- [22] K. Saito, A. Mitsui, I.W. Sumardika, Y. Yokoyama, M. Sakaguchi, E. Kondo, PLOD2-driven IL-6/STAT3 signaling promotes the invasion and metastasis of oral squamous cell carcinoma via activation of Integrin beta1, *Int. J. Oncol.* 58 (6) (2021) 29.
- [23] D.E. Muench, A. Olsson, K. Ferchen, G. Pham, R.A. Serafin, S. Chutipongtanate, et al., Mouse models of neutropenia reveal progenitor-stage-specific defects, *Nature* 582 (7810) (2020) 109–114.
- [24] X. Sheng, Y. Li, Y. Li, W. Liu, Z. Lu, J. Zhan, et al., PLOD2 contributes to drug resistance in laryngeal cancer by promoting cancer stem cell-like characteristics, *BMC Cancer* 19 (1) (2019) 840.
- [25] F.J. Garcia-Leon, R. Garcia-Esteva, A. Romero-Tabares, J. Gomez-Millan Borrachina, Treatment of advanced laryngeal cancer and quality of life. Systematic review, *Acta Otorrinolaringol. Esp.* 68 (4) (2017) 212–219 (Engl Ed).
- [26] P. Dwivedi, S. Chutipongtanate, D.E. Muench, M. Azam, H.L. Grimes, K.D. Greis, SWATH-proteomics of ibrutinib's action in myeloid leukemia initiating mutated G-CSFR signaling, *Proteom. Clin. Appl.* 14 (5) (2020), e1900144.
- [27] P. Dwivedi, D.E. Muench, M. Wagner, M. Azam, H.L. Grimes, K.D. Greis, Time resolved quantitative phospho-tyrosine analysis reveals Bruton's Tyrosine kinase mediated signaling downstream of the mutated granulocyte-colony stimulating factor receptors, *Leukemia* 33 (1) (2019) 75–87.
- [28] V.M. Kumbar, U.M. Muddapur, K.G. Bhat, H.R. Shwetha, M.S. Kugaji, M.R. Peram, et al., Cancer stem cell traits in tumor spheres derived from primary laryngeal carcinoma cell lines, *Contemp. Clin. Dent.* 12 (3) (2021) 247–254.
- [29] M.W. Sim, P.T. Grogan, C. Subramanian, C.R. Bradford, T.E. Carey, M.L. Forrest, et al., Effects of peritumoral nanoconjugated cisplatin on laryngeal cancer stem cells, *Laryngoscope* 126 (5) (2016) E184–E190.
- [30] L. Fu, J. Fan, S. Maity, G. McFadden, Y. Shi, W. Kong, PD-L1 interacts with Frizzled 6 to activate beta-catenin and form a positive feedback loop to promote cancer stem cell expansion, *Oncogene* 41 (8) (2022) 1100–1113.
- [31] T. Yang, X. Shu, H.W. Zhang, L.X. Sun, L. Yu, J. Liu, et al., Enolase 1 regulates stem cell-like properties in gastric cancer cells by stimulating glycolysis, *Cell Death Dis.* 11 (10) (2020) 870.
- [32] Y. Tao, H. Shen, Y. Liu, G. Li, Z. Huang, Y. Liu, IL-23R in laryngeal cancer: a cancer immunoeediting process that facilitates tumor cell proliferation and results in cisplatin resistance, *Carcinogenesis* 42 (1) (2021) 118–126.
- [33] J.F. Lu, D. Pokharel, M. Bebawy, MRP1 and its role in anticancer drug resistance, *Drug Metab. Rev.* 47 (4) (2015) 406–419.
- [34] R.W. Robey, K.M. Pluchino, M.D. Hall, A.T. Fojo, S.E. Bates, M.M. Gottesman, Revisiting the role of ABC transporters in multidrug-resistant cancer, *Nat. Rev. Cancer* 18 (7) (2018) 452–464.
- [35] A. Kumar, V. Jaitak, Natural products as multidrug resistance modulators in cancer, *Eur. J. Med. Chem.* 176 (2019) 268–291.
- [36] F. Balthes, V. Pfeifer, K. Silbermann, J. Caspers, K. Wantoch von Rekowski, M. Schlesinger, et al., beta1-Integrin binding to collagen type 1 transmits breast cancer cells into chemoresistance by activating ABC efflux transporters, *Biochim. Biophys. Acta Mol. Cell Res.* 1867 (5) (2020), 118663.