The Antitumor Activity of Pseudoginsengenin DQ Against Hypopharyngeal Cancer Cells via Targeting HIF-1 α -GLUT1 Pathway

Sanchun Wang

Jilin University Second Hospital <https://orcid.org/0000-0003-1497-1193>

Yu Cai

Jilin University Second Hospital

Qingjie Feng

Jilin University Second Hospital

Jing Gao

Changchun Institute of Applied Chemistry Chinese Academy of Sciences: Chang Chun Institute of Applied Chemistry Chinese Academy of Sciences

Bo Teng (✉ tengbo@jlu.edu.cn)

Jilin University Second Hospital <https://orcid.org/0000-0003-1231-3680>

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Abstract

Background

The ginsenosides have been reported to possess a variety of biological activities. Synthesized from the ginsenoside Protopanaxadiol (PPD), the octanone Pseudoginsengenin DQ (PDQ) may have stronger pharmacological effects as a secondary ginsenoside. Nevertheless, its antitumor activity and molecular mechanism against hypopharyngeal cancer cells remains unclear.

Methods

Cell Counting Kit-8, cell cycle assay and cell apoptosis assay were conducted to detect FADU cells proliferation, cell phase and apoptosis. The interactions between PDQ and HIF-1 α were investigated by a molecular docking study. The expression of HIF-1 α , GLUT1, apoptosis related proteins was tested by western blotting, direct stochastic optical reconstruction microscopy (dSTORM) and qRT-PCR. Glucose uptake assay was used to assess the glucose uptake capacity of FADU cells.

Results

PDQ was found to suppress the proliferation, reduce glucose uptake, induce the cell cycle arrest and apoptosis of FaDu cells. Molecular docking study demonstrated that PDQ could interact with the active site of HIF-1 α . PDQ decreased the expression and mRNA levels of HIF-1 α and its downstream factor GLUT1. Moreover, dSTORM results showed that PDQ reduced GLUT1 expression on the cell membrane but also inhibited its clustering.

Conclusion

Our work elucidated that the antitumor effect of PDQ is related to its downregulation of HIF-1 α -GLUT1 pathway, suggesting that PDQ could be a potential therapeutic agent for hypopharyngeal cancer treatment.

Introduction

Hypopharyngeal carcinoma is one of the most challenging head and neck malignancies in treatment, and is still one of the worst prognosis head and neck malignant tumors[1]. The 5-year overall survival rate is only 30% – 35%[2]. Surgery followed by radiotherapy has been the traditional treatment for advance-stage patients[3], but total laryngectomy will make them lose voice function. Although the strategy of organ preservation with chemotherapy can achieve similar survival rate as surgery plus radiotherapy and retain laryngeal function[4], chemotherapy has some kinds of acute and late toxicities. Therefore, it is

indispensable to seek more effective and novel natural drugs with low side effects to treat hypopharyngeal cancer.

Ginseng has long been used as a general tonic to strengthen the body immunity and prolong life in traditional Chinese medicine[5]. As the main effective constituents of ginseng, ginsenosides possess a wide spectrum of pharmaceutical activities including central nervous regulation, immune function enhancement, cardiovascular health protection, antiaging and antitumor [6]. Hypoxia-inducible factor 1 (HIF-1) is an essential transcription factor contributing to cellular oxygen sensing and adaptation to hypoxia. The enzymes of glucose metabolism, including glucose transporters 1 (GLUT1) are regulated by HIF-1, the expression of these genes regulated by HIF1 alters the intracellular biological functions like glucose uptake and energy production[7, 8]. which is associated with cancer cells proliferation and poor prognosis in tumors[9, 10]. Studies have shown that ginsenoside can inhibit the growth of liver or lung cancers by inhibiting hypoxia-inducible factor-1 α (HIF-1 α) mediated glucose metabolism[11, 12]. With the deepening of research, it is found that secondary ginsenosides and aglycones produced by degradation of ginsenosides have stronger pharmacological effects[13]. Pseudoginsengonin DQ (PDQ) is a kind of octylon ginsenoside synthesized from protopanaxadiol saponins by oxidation and cyclization under the acidic condition[14]. Recent studies have demonstrated that PDQ can be used to improve aconitine-induced arrhythmia, or cisplatin-induced acute kidney injury[15, 16]. However, its antitumor activity against hypopharyngeal carcinoma cells and the underlying mechanism have rarely been investigated.

Previous studies usually used classical methods such as Western blotting, real-time RT-PCR and flow cytometry to elucidate the problems similar to the above. These methods are based on the average of the ensemble level of related molecules. In order to reflect the spatial distribution and structural arrangement of signal proteins at the single-molecule level, one of the super-resolution imaging techniques, direct stochastic optical reconstruction microscopy (dSTORM)[17], needs to be utilized. dSTORM relies on fluorophores that can be switched between a bright on and a dark off state. Only a few molecules are randomly excited to the bright on state and their positions are recorded. By repeating this process, a reconstructed super-resolution image is finally obtained by accumulating the precise locations of each detected molecule. This approach allows direct observation of protein distribution with a resolution of decades of nanometer[18, 19]. Thus, the biochemical methods and dSTORM are complementary to each other, and combining them is benefit for acquiring both overall and single-molecule information of PDQ's antitumor mechanism.

In this work, we investigated the antitumor effects of PDQ on human hypopharyngeal carcinoma FaDu cells. PDQ is found to suppress cell proliferation, and induce cell apoptosis and trigger the cell cycle arrest. Furthermore, molecular docking was performed to identify HIF-1 α as the antitumor target of PDQ in hypopharyngeal carcinoma. Ultimately, the underlying molecular mechanism of PDQ's antitumor effect through the inhibition of HIF-1 α -GLUT1 pathway was elucidated by classical biotechnologies and super-resolution fluorescence microscopy.

Materials And Methods

Cell culture

FaDu cell line was purchased from the Shanghai Institute of Biological Sciences and cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 100 µg/ml penicillin/streptomycin (P/S; Invitrogen). Cells were maintained at 37°C with 5% CO₂.

Cell cytotoxicity assay of PDQ

PDQ was obtained from the School of Pharmaceutical Sciences of Jilin University, Changchun, China. Logarithmic growth phase of FaDu cells were seeded in 96-well plates at 5000 cells/well and cultured overnight. Then cells were treated with different concentrations of PDQ (40, 60, 80, 100, 120, 140, 160 and 180 µmol/L) for 24 h, 48 h and 72 h. Cells treated with the same amount of anhydrous ethanol (PDQ=0 µmol/L) were used as control. The final concentration of ethanol was 0.1%. Five parallel samples were set for each concentration. After that, 10 µL of Cell Counting Kit 8 (CCK8; Beyotime) was added to every well and the cells were incubated for 2 h at 37°C with 5% CO₂. Finally, the absorbance at 450 nm of every well was read by a Bio-Rad microplate reader (model 630; Hercules, CA, USA).

Safety detection of PDQ in vivo

Ten female BALB/c mice (18–22 g) were purchased from the Animal Center of Norman Bethune Medical College of Jilin University (Changchun, China). All animals were maintained under standardized laboratory conditions (12 h light/dark cycle beginning at 08:00 a.m, temperature 22–25 °C, relative humidity 50–70%), with free access to food and water. The mice were randomly divided into control and PDQ group (5 mice/group). Mice in PDQ group were gavaged (P.O) with 40 mg/kg PDQ (suspended in 0.05% carboxymethylcellulose sodium) for one time while mice in the control group were treated with equal carboxymethylcellulose sodium. All mice general clinical conditions were daily observed for a 2-week follow-up period. Mice were sacrificed by cervical vertebra dislocation at day 14. The whole blood was collected from the orbit and the serum was separated from the blood by centrifugation (3500 rpm, 15 min, 4 °C). The hematopoietic and biochemical analysis were determined by automatic hematology analyser (HC2200, Meiyilinm, China). Heart, lungs, liver, spleens and kidneys were rapidly collected and cut into 4–5 µm-thick sections, embedded in paraffin, stained with Hematoxylin - Eosin and examined with Nikon TE 2000 fluorescence microscope (Nikon, Japan). All animals in this study were handled according to a protocol approved by the Institutional Animal Care and Use Committee of Jilin University (No. 2016135).

Glucose uptake assay

Cells (3×10^5 cells/well) were plated in 6-well plates. After the cells were cultured for 24h with different concentration of PDQ, the supernatant of culture medium was collected to examine the concentration of glucose using the Glucose Assay Kit (BestBio, BB-4731-1). The glucose concentration was quantified by

the absorbance at 505nm by a plate reader (BioTek), and normalized by protein concentration of respective samples.

Cell cycle analysis by flow cytometry

To analyze cell cycle, FaDu cells at the logarithmic growth phase were harvested and washed with ice-cold PBS, then fixed with ice-cold 70%-75% ethanol at -4°C overnight. After that, cells were washed with cold PBS, subsequently incubated with 100 ng/mL RNase A for 30 min at 37°C , and then filtered by 400-mesh screen. Next, cells were stained with 10 $\mu\text{g/mL}$ PI for 30 min at 4°C in dark. Finally, samples were tested by flow cytometry with FACS Diva Software (Becton Dickinson) to analyze DNA content and light scattering.

Cell apoptosis analysis by flow cytometry and TUNEL

To analyze cell apoptosis, FaDu cells were plated in 6-well plates and treated with different concentration of PDQ (60, 100 and 140 $\mu\text{mol/L}$) or with the same amount of anhydrous ethanol. After 24 h, cells were harvested and labeled with Annexin V-FITC-PI Apoptosis Detection Kit (BD Biosciences, Beijing, China) according to the manufacturer's instruction and analyzed by flow cytometry (Becton Dickinson).

Moreover, immunofluorescence staining of TUNEL was also performed to detect cell apoptosis. Cells were cultured at high confluence of 80%-90% and treated with different concentration of PDQ (0, 60, 100, and 140 $\mu\text{mol/L}$). After 24 h incubation, cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.5% Triton X-100 for 10 min. Next, cells were incubated with TUNEL (Promega Corp., Madison, WI, USA) at 37°C for 30 min, followed by incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (Cell Signaling Technology) for 1 h. Finally, the samples were observed by a Zeiss 510 Meta laser scanning confocal microscope (Zeiss). The exposure time of all pictures was 200 ms.

Molecular docking

The software of Schrödinger Suites (2015) was used to perform the molecular docking between PDQ and protein receptors. The crystal structures of proteins were obtained from the Protein Data Bank. The three-dimensional (3D) structure of PDQ was drawn via 'Maestro Elements' in the software, and its bond angle and order was assigned by 'Ligand Preparation'. Protein receptors and PDQ were docked with 'Glide Docking', their docking results were visualized through the PyMOL Molecular Graphics System.

Quantitative Real-Time RT-PCR

FaDu cells were cultured in 96-well plates and treated with different concentration of PDQ (60, 100 and 140 $\mu\text{mol/L}$) or with the same amount of anhydrous ethanol. After incubation for 24 h, total RNA was extracted using TRIzol reagent (Invitrogen, USA). cDNA was synthesized by reverse transcription ReverTra Ace[®] qPCR RT Master Mix with gDNA Remover (TOYOBO Co., LTD) and subjected to real-time PCR with gene-specific primers in the presence of Cybergreen (Applied Biosystems). Experiments were performed at least three times with duplicate replicates. Relative abundance of mRNA was calculated by normalization

to GAPDH. Primer pairs used here were as follows (purchased from Genecopoeia Co., Ltd.): GAPDH: forward 5'- TTCTTTTTCGTCGCCAGCCGAG -3', reverse 5'- CCAGGCGCCCAATACGACCAAA -3'; GLUT1: forward 5'- CTGGCATCAACGCTGTCTTC -3', reverse 5'- GCCTATGAGGTGCAGGGTC -3'; HIF-1 α : forward 5'- AGACAAAGTTCACCTGAGCC -3', reverse 5'- GGGAGCTAACATCTCCAAGTCT -3'.

Western blotting

FaDu cells were treated with different concentration of PDQ (60, 100 and 140 μ mol/L) or with the same amount of anhydrous ethanol. After incubation for 24 h, cells were harvested and subjected to SDS-PAGE, and processed per antibody manufacture's instruction (BD Biosciences). The primary antibodies were used as follows: anti-HIF-1 α (1:200 dilution, Santa Cruz, sc-13515), anti-GLUT1 (1:200 dilution, Santa Cruz, sc-377228), anti-Bcl-2 (1:1000 dilution; Bioss, bsm-52304R.), anti-Bax (1:1000 dilution; Bioss, bsm-52316R.), anti-Caspase9 (1:800 dilution; Proteintech, 10380-1-AP.), and anti-Caspase3 (1:1000 dilution; Proteintech, 19677-1-AP.), and GAPDH (1:10000 dilution; Abcam, Inc.) Following incubation with the corresponding secondary antibodies, the signals were developed using an Amersham ECL Plus Western Blotting Detection System (GE Healthcare). Data were presented as relative protein levels which were normalized to GAPDH, and the ratio of control samples was taken as 100%.

dSTORM imaging

Cells were passaged on a pre-cleaned standard microscope slide (22 mm $\times$ 22 mm, Fisher) in the dish and treated with 100 μ M PDQ or left untreated. After 24 h, the solution was removed and cells were rinsed with PBS for three times. Subsequently, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, and blocked with 3% Bovine Serum Albumin for 30 min. Then cells were incubated with anti-human GLUT1 antibodies (2 μ g/ml in 3% BSA; Santa Cruz, sc-377228) overnight at 4 $^{\circ}$ C, and washed with PBS. At last, cells were stained with Alexa Fluor 647 goat anti-mouse IgG antibodies (2 μ g/ml in 3% BSA; Invitrogen, A-21235) for 1 h. Before imaging, 50 μ l imaging buffer containing Tris (50 mM, pH 8.0), NaCl (10 mM), glucose (10% w/v), glucose oxidase (500 μ g /ml; Sigma), catalase (40 μ g/ml; Sigma) and β -mercaptoethanol (1% v/v; Sigma) was dropped on a large microscope slide (24 mm $\times$ 50 mm, Fisher), and the small slide where cells were seeded was covered on the large one and sealed with nail polish.

dSTORM imaging was performed on a Nikon Ti-E microscope with a 100 $\times$ 1.49 NA TIRF lens (Nikon, Japan). The sample was illuminated in total internal reflection fluorescence (TIRF) mode. The 640 nm laser was used to excite fluorophores of Alexa Fluor 647, and the 405 nm laser was used to increase the number of on-state fluorophores by carefully controlling its irradiation intensity (<0.1 kW/cm 2). All images were captured by a cooled EMCCD (Andor Ixon Ultra 888). 5000 images were collected for each cell with an internal time of 25 ms between frames to reconstruct a super-resolution image. TetraSpeck microspheres of 100 nm (Invitrogen,) were embedded in the sample to correct x-y drift, and a focus lock was used to correct z drift.

dSTORM data analysis

Raw dSTORM image sequences were analyzed by ThunderSTORM[20], a free available plug-in in Image J, to obtain a reconstructed dSTORM image. To characterize the spatial distribution of GLUT1 on cell membranes, SR-Tesseler analysis method was used as previously reported[21]. Firstly, a file including the coordinates, intensity and sigma of localizations was loaded in the program. The reconstructed image was shown and an ROI of cell could be selected to analyze (Figure S1a). Secondly, bisectors between two closest localizations were drawn, and the ROI was segmented into many polygons with different number of localizations (Figure S1b). The localization density of a polygon was defined as δ_{i1} , and the average localization density of the total ROI was δ_0 . If $\delta_{i1} > \delta_0$, localizations in this polygon were picked up to create an 'object' (Figure S1c). Similarly, the localization density of an object was set as δ_{i2} , and the average of all objects was δ_1 . Thirdly, objects satisfied the requirement of $\delta_{i2} > \delta_1$ were extracted as clusters (Figure S1d). Finally, the area, the number of localizations, coordinates and morphological parameters of each cluster were computed and exported.

Statistical Analysis

Data were expressed as mean $\pm$ SD (standard deviation). All statistical analysis was performed using SPSS version 22.0 (IBM, Chicago, Illinois, USA). Group means were compared by one-way analysis of variance (ANOVA), and P values <0.05 were considered significant in all cases. In addition, two-tailed unpaired t-test was used for the statistical analysis of dSTORM data, and P values <0.05 were considered significant.

Results

PDQ inhibits the proliferation of FaDu cells

We firstly carried out CCK8 assay to assess the cytotoxicity of PDQ in FaDu cells. As shown in Figure 1, cells were exposed to different concentration of PDQ for 24 h, 48 h and 72 h. The plots indicated that the cell survival rate was the lowest and the IC50 value (100 $\mu\text{mol/L}$) was minimal after exposure for 24 h. For 48 h and 72 h, the survival rate increased slightly. Moreover, PDQ significantly inhibited the proliferation of FaDu cells when the concentration of PDQ was higher than 60 $\mu\text{mol/L}$ and the inhibition was dose-dependent. The results suggested that PDQ inhibited the proliferation of hypopharyngeal cancer cells in a dose-dependent but time-independent manner. Accordingly, we applied the effective PDQ concentration in the range of 60-140 $\mu\text{mol/L}$ and 24 hours' treatment in subsequent experiments.

In order to exclude that the safety of PDQ and systematic effect may lead to cell growth inhibition, we performed in vivo experiment. No mice died during the 14-day follow-up period. Hemogram assay indicated that the white blood cell (WBC), red blood cell (RBC), lymphocyte (LYM) and monocyte (MONO) counts had no significant differences between PDQ and control groups. The levels of the serum biochemical markers ALT, AST, BUN and CRE had no significant differences between those two groups, neither (Table S1). In order to evaluate the effect of PDQ on major organs, we analyzed the pathological pattern of heart, liver, spleen, lung and kidney, no major histological changes were observed in the PDQ

group compared to the control group (Figure S1). The results verified that PDQ had no side effects on animals.

PDQ triggers the cancer cell cycle arrest

Cell proliferation depends on the cell cycle, and the cell cycle is an accurately regulated process that allows the cell to duplicate and grow. Since PDQ can inhibit the proliferation of FaDu cells, we wondered whether PDQ could affect the cell cycle. To confirm this possibility, we analyzed the distribution of cell cycle by flow cytometry in control and 100 $\mu\text{mol/L}$ PDQ-treated cells. The results showed that G0/G1 phase cells increased significantly whereas S and G2/M phase cells occupied less proportion after PDQ treatment (Figure 2). This finding validated our hypothesis that PDQ caused the cell cycle arrest in G0/G1 phase.

PDQ induces the apoptosis of FaDu cells

To evaluate the effects of PDQ on apoptosis, Annexin V-FITC and PI double staining was investigated in PDQ-treated FaDu cells by flow cytometry. The results revealed that the proportion of both early and late apoptotic cells remarkably increased in a dose-dependent way (Figure 3a). Specifically, the rate of apoptotic cells increased from $5.06 \pm 1.94\%$ in control group to $31.67 \pm 4.5\%$ of the cells treated with 100 $\mu\text{mol/L}$ PDQ for 24 h and $56.37 \pm 3.80\%$ of the cells treated with 140 $\mu\text{mol/L}$ PDQ for 24 h (Figure 3b).

Subsequently, immunofluorescence with TUNEL staining was performed to detect the morphology of apoptotic FaDu cells and further support the apoptosis effect of PDQ. Apoptotic cells usually exhibit nuclear condensation and DNA fragmentation, and the fragmented DNA in nuclei can be observed as green fluorescence signals under the fluorescence microscopy after TUNEL staining. As illustrated in Figure 4a, cells with TUNEL positive signal increased gradually as PDQ concentration increased. Especially for treatment with 100 or 140 $\mu\text{mol/L}$ PDQ for 24 h, the percentage of apoptotic cells was $50.67 \pm 3.05\%$ and $71.00 \pm 1.73\%$ respectively (Figure 4b), which was much higher than the control group ($3.66 \pm 1.15\%$). Collectively, both flow cytometry and TUNEL assay demonstrated that PDQ significantly triggered the apoptosis of FaDu cells in a dose-dependent manner.

To determine the effects of PDQ treatment on the expression of apoptosis-related protein, the level of Bcl-2, Bax, Caspase9, and Caspase3 were examined by western blot assays. The PDQ treatment significantly increased the expression levels of the apoptotic protein Bax and suppressed the expression of the anti-apoptotic protein Bcl-2 in a dose dependent manner. Moreover, PDQ up-regulated the expression of apoptosis-associated enzymes Caspase-9 and Caspase-3 in a concentration-dependent manner as well (Figure 5a and b). These results demonstrated that PDQ induced apoptosis was achieved by the regulation of apoptosis-related proteins.

Molecular docking between PDQ and HIF-1 α

PDQ can suppress cell proliferation, lead to the cell cycle arrest and induce cell apoptosis. We had verified its antitumor activity against hypopharyngeal cancer cells, and next we wanted to explore the underlying

mechanisms of its action. To determine whether PDQ is associated with HIF-1 α or GLUT1, we performed a molecular docking study. The chemical structure of PDQ is shown in Figure 6a. After the precise docking of PDQ with these two proteins, we found that PDQ has a high docking score with HIF-1 α , which is -7.3. As shown in Figure 6b, the C-25 hydroxyl group of PDQ interacts with the residue His378 through a hydrogen bond in the active site of HIF-1 α . The above results indicated that PDQ has high affinity with HIF-1 α and displays a key molecular docking interaction with contiguous amino acid in the active site of HIF-1 α , which hinted us that the antitumor effect of PDQ might be related to HIF-1 α signalling pathway.

PDQ downregulates HIF-1 α -GLUT1 pathway

HIF-1 α is a ubiquitously expressed, master regulator of genes that allows adaptation to hypoxic conditions[22, 23]. Its target genes include VEGF, erythropoietin, LDHA, GLUT1 and other factors critical to vascularization, metabolic regulation, cell multiplication and survival[24]. As HIF-1 α and GLUT1 are considered as intrinsic hypoxia markers, we selected GLUT1, one of the important HIF-1 α downstream effectors, as a study object. To further verify that PDQ is interrelated with HIF-1 α -GLUT1 pathway in FaDu cells, we analyzed the mRNA expression of HIF-1 α and GLUT1 using qRT-PCR assay. FaDu cells were treated with different concentration of PDQ (0, 60, 100 and 140 μ mol/L). Figure 7 reveals that PDQ significantly inhibited HIF-1 α expression at the mRNA level in a concentration-dependent fashion. The mRNA level of GLUT1 reduced markedly when the concentration of PDQ increased to 100 μ mol/L or even to 140 μ mol/L. The results indicated that high concentration PDQ treatment significantly decreased the mRNA expression of HIF-1 α and GLUT1.

Subsequently, to further explain the mechanism of PDQ targeting HIF-1 α -GLUT1 pathway in hypopharyngeal cancer cells, the protein levels of HIF-1 α and GLUT1 were explored by Western blot assay. As observed in Figure 8, PDQ treatment resulted in the decrease of both proteins. Especially for 100 μ mol/L and 140 μ mol/L PDQ groups, HIF-1 α fell down to about 0.47 and 0.16 times of the control group, and GLUT1 dropped to 0.75 and 0.45 times, which were in agreement with the results of RT-PCR analysis.

Since the expression levels of HIF-1 α and GLUT1 decreased by PDQ treatment and they also affect the energy supply of cancer cells, we investigated the glucose uptake of FaDu cells in response to PDQ treatment. The cells were treated with different PDQ concentrations for 24 h. Compared to the control group, PDQ significantly inhibited glucose uptake when the concentration of PDQ increased to 100 μ mol/L or 140 μ mol/L (Figure 9). This demonstrated that PDQ inhibited glucose uptake significantly and suppressed cell proliferation by reducing the energy supply. Taken together, the above results demonstrated that PDQ downregulates HIF-1 α -GLUT1 signalling pathway, which may contribute to the antitumor activity of PDQ.

In addition to the expression level of HIF-1 α -GLUT1 pathway, to further investigate whether PDQ affects the detailed distribution of these tumor-associated proteins, we performed dSTORM imaging. As GLUT1 is a vital downstream factor of HIF-1 α and mainly distributes on the cell membrane, it is very suitable to be observed by dSTORM. Therefore, FaDu cells were treated with 100 μ mol/L PDQ or left untreated, and

then the labelled samples were illuminated under the 647 nm laser. As shown in Figure 10a-d, GLUT1 on the cell membrane decreased significantly after PDQ treatment. To accurately assess the effect of PDQ on the expression levels of GLUT1, we quantitatively analyzed the number of localizations (Figure 10e), which was proportional to the amounts of proteins. The result showed that the localization number of GLUT1 fell from 543 ± 12 per μm^2 to 326 ± 9 per μm^2 with PDQ treatment, which was consistent with the Western blot assay (Figure 7), and thus it confirmed again the downregulation of PDQ on GLUT1.

More intriguingly, we found that PDQ treatment changed the morphology and spatial distribution of GLUT1 on the cell membrane. To quantitatively analyze its distribution pattern, we used SR-Tesseler method[25] to characterize the potential clusters. This method is based on Euclidean distances to divide regions and multiple thresholding of localization density to select qualified points (Figure S1, see Materials and methods for detail). We first analyzed the cluster number of GLUT1 per unit. It decreased almost a half after adding PDQ (Figure 10f). We next extracted the information of cluster diameter. In control cells, clusters with a diameter ranged from 200 nm to 300 nm occupied the greatest percentage, and clusters with the diameter of 100-200 nm and 300-400 nm were the second and third most abundant respectively (Figure 10g). However, the clusters got very small after PDQ treatment. Clusters with 200-400 nm were less than ten, and there were no clusters with the diameter more than 400 nm. Only 50-200 nm clusters were found on the membrane. We also explored the molecular composition of clusters. Only clusters consisting of 60-100 localizations on PDQ-treated cell membranes were more than those on control cell membranes (Figure 10h). For other large clusters containing more than 100 localizations, the control group had twice or even several times more than the PDQ-treated group. Collectively, these results indicated that PDQ regulates the redistribution of GLUT1 clusters on the membrane. It exerts an inhibiting effect on HIF-1 α -GLUT1 pathway through not only reducing the expression levels of HIF-1 α and GLUT1 but also disrupting the cluster formation of the downstream signalling protein GLUT1.

Discussion

Hypopharyngeal carcinoma is one of the worst prognosis head and neck malignant tumors. Laryngectomy could make patients lose voice function, and conventional radiotherapy or chemotherapy shows poor antitumor and significant toxicity and side effects. Owing to the strong pharmaceutical properties and low side effects, ginseng has become a good choice in the treatment of cancer. Pseudoginsenoside DQ is an important bioactive ingredient that is synthesized from protopanaxadiol saponins. In addition to its role in the treatment of coronary heart disease, myocardial ischemia and arrhythmia, its antitumor effect on hypopharyngeal cancer and the underlying molecular mechanism require further clarification.

In the present study, we firstly confirmed that PDQ can suppress the growth and viability of FaDu cells (Fig. 1). By flow cytometry, Western blot and TUNEL assay, we next found that PDQ can induce the hypopharyngeal cancer cell cycle arrest (Fig. 2) and apoptosis (Fig. 3, 4, 5). Together, we revealed that PDQ has an antitumor effect on hypopharyngeal cancer cells, and its antitumor activity is achieved by the molecular mechanisms of inhibiting proliferation and inducing apoptosis. The results were consistent

with previous medical and experimental researches about ginsenosides in other cancers, such as hepatocellular carcinoma[26], cervix carcinoma[27], colorectal cancer[28], prostate cancer[29] and breast cancer[30].

Hypoxia is a common feature of malignancy and particularly of solid tumors, which is able to accelerate tumor invasiveness and metastasis[31, 32]. HIF-1 α is the principal mediator during the cellular adaptive response to hypoxia[22]. It regulates multiple aspects of tumorigenesis including proliferation, differentiation, angiogenesis, energy metabolism, and metastasis[33, 34], thereby influencing the expression of target proteins, such as VEGF, erythropoietin, GLUT1, glycolytic enzymes and so forth[24, 35]. Among them, GLUT1 can mediate glucose transport to meet the energy requirements of tumor cells. HIF-1 α and GLUT1 are considered as intrinsic hypoxia markers and have been studied the most in various tumors[36–40]. Their respective high expression and the correlation between their expression have been identified in many types of tumor cells and biopsy tissue samples, which may promote tumor progression and lead to a poor survival rate and prognosis[37–41]. To investigate whether HIF-1 α or GLUT1 is the target molecules of PDQ, we performed the molecular docking of PDQ with those two proteins. The results showed that PDQ can interact with HIF-1 α with high molecular binding force, suggesting that HIF-1 α is very likely to be the main target of PDQ.

Since the molecular docking experiment showed the interaction between PDQ and HIF-1 α , we evaluated the mRNA and protein levels of HIF-1 α and GLUT1. Both of their mRNA and protein levels decreased significantly after PDQ treatment (Fig. 7, 8), suggesting that PDQ's antitumor effect may be associated with inhibiting HIF-1 α -GLUT1 pathway. This antitumor mechanism was consistent with other studies. For example, Chen et al found that inhibiting the expression of HIF-1 α decreased the GLUT1 expression level, and thus caused a reduction of the tumor volume and weight of LOVO cell line xenografts[42]. Some studies also have proposed that combined inhibition of HIF-1 α and GLUT1 could be a promising cancer therapeutic strategy[43–46]. Our work demonstrated that PDQ is just one of the first choice drugs that realize this antitumor effect.

Not only the overall expression level, the detailed distribution of GLUT1 on hypopharyngeal cancer cell membrane was also revealed by dSTORM imaging (Fig. 10). With PDQ treatment, the localization number of GLUT1 decreased significantly, which was in agreement with the results of Western blot analysis. Moreover, PDQ blocked the formation of GLUT1 clusters, and even if there were still clusters on the membranes, the cluster size and the number of GLUT1 molecules in clusters reduced sharply. The clustering of GLUT1 on cancer cell membranes has been studied before[18]. These protein clusters are beneficial to the rapid response of receptors to signals and signal transmission. Thus, the weakening of GLUT1 clusters further verified that PDQ could target HIF-1 α -GLUT1 pathway by inhibiting both the expression and clustering of GLUT1. The main text.

Conclusions

In conclusion, combined classical biochemistry methods with super-resolution fluorescence imaging, we elucidate the antitumor activities of PDQ on human hypopharyngeal cancer cells and uncover its antitumor mechanism. PDQ can inhibit cell proliferation, induce cell apoptosis and trigger the cell cycle arrest at G0/ G1 phase. More importantly, our findings indicate that HIF-1 α is the target molecule of PDQ and PDQ's antitumor effect is associated with suppressing HIF-1 α -GLUT1 pathway by downregulating the expression of HIF-1 α and GLUT1 as well as disrupting the clustering of GLUT1. The current work reveals the role of PDQ in blocking the energy supply of cancer cells, which provides new insights into the molecular mechanism of ginsenoside's antitumor effect. In the future, we will explore more target molecules of PDQ in cancer cells and perform animal experiments to evaluate its therapeutic effects for further developing it as a useful drug to treat hypopharyngeal carcinoma.

Abbreviations

PDQ: Pseudoginsengonin DQ; HIF-1 α : hypoxia-inducible factor-1 α ; GLUT1: glucose transporters 1; dSTORM: direct stochastic optical reconstruction microscopy.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

SCW and YC: Contributed to study design, performed lab work, drafted and revised the paper. QJF and JG: Contributed to the experiments and commented final version of manuscript. BT: Helped in conceptualization, data curation, manuscript writing and resources. All authors read and approved the final manuscript.

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Availability of data and materials

Please contact the corresponding author for all data requests.

Ethics approval and consent to participate

The study was approved by the Institutional Animal Care and Use Committee of Jilin University (No. 2016135).

Consent for publication

All authors have read and approved the final manuscript.

Competing interests

There are no conflicts to declare.

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Figures

Figure 1

The effect of PDQ on the proliferation of FaDu cells. (a) The plots show the survival rate of cells treated with different concentration of PDQ for 24 h, 48 h, and 72 h. (b) The survival rate of cells treated with 100

μmol/L PDQ for different time. Data are shown as mean ± S.D., ***p< 0.001, compared to control, n = 3.

Figure 2

Flow cytometric detection of the cell cycle distribution. (a, b) The distribution of cell cycle in control group (a) and 100 μmol/L PDQ-treated group (b). (c) The percentage of cells in each phase without or with PDQ treatment. Data are shown as mean ± S.D., **p< 0.01, compared to control, n = 3.

Figure 3

Flow cytometric analysis of the apoptosis of FaDu cells. (a) Annexin V-FITC and PI double staining to test the cell apoptosis without or with the treatment of 60, 100, and 140 μmol/L PDQ for 24 h. The Q1-Q4 quadrant represent necrotic cells, late apoptotic cells, early apoptotic cells and viable cells, respectively.

(b) The percentage of total, early and late apoptotic cells treated with the different concentration of PDQ. Data are shown as mean $\pm$ S.D., * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to control, $n = 3$.

Figure 4

TUNEL analysis of the apoptosis of FaDu cells. (a) Confocal images of cells under the bright field and TUNEL staining without or with the treatment of different concentration of PDQ. (b) The percentage of apoptotic cells from TUNEL analysis. Data are shown as mean $\pm$ S.D., ** $p < 0.01$, compared to control, $n = 3$. Scale bars are 50 μ m.

Figure 5

Western blot analysis of apoptosis-associated proteins. (a) The expression of apoptosis-associated proteins with the different concentration of PDQ treatment. (b)The relative protein expression is calculated after correction for the GAPDH loading control. Data are shown as mean $\pm$ S.D., * $p < 0.05$, ** $p < 0.01$, compared to control, $n = 3$.

Figure 6

Molecular docking of ginsenoside PDQ and HIF-1 α . (a) The chemical structure of 24 (S)-PDQ. (b) Molecular docking of PDQ in the active site of HIF-1 α . The C-25 hydroxyl group in PDQ structure forms a hydrogen bond with His378 residue of HIF-1 α (PDB ID: 4H6J). The docking score is -7.3.

Figure 7

Quantitative RT-PCR analysis of HIF-1 α and GLUT1 transcription with PDQ treatment. The relative levels of their mRNA under the different concentration of PDQ are displayed in a histogram. Data are shown as mean $\pm$ S.D., * p < 0.05, ** p < 0.01, *** p < 0.001, compared to control, n = 3.

Figure 8

(a) Western blot analysis of the expression levels of HIF-1 α and GLUT1 with PDQ treatment and quantitation of the data by Image J. (b) The relative protein expression is calculated after correction for the GAPDH loading control. Data are shown as mean $\pm$ S.D., * p < 0.05, ** p < 0.01, compared to control, n = 3.

Figure 9

Glucose uptake analysis of relative levels of glucose consumption under the different concentration of PDQ. Data are shown as mean $\pm$ S.D., * $p < 0.05$, ** $p < 0.01$, compared to control, $n = 3$.

Figure 10

PDQ affected the distribution of GLUT1 and weakened its clustering on the membrane. (a, c) dSTORM imaging of GLUT1 on FaDu cell membrane without treatment (a) or with 100 μ M PDQ treatment (c). (b, d) Magnified regions of GLUT1 corresponding to the white box in (a) and (c). Scale bars, 5 μ m in (b) and (d), 1 μ m in (a) and (c). (e) The number of GLUT1 localizations per μ m² on control and PDQ-treated cell membranes. (f) The number of clusters per μ m². (g) The distribution of cluster diameter. (h) The distribution of localization number per cluster. Data shown in (e-h) are means $\pm$ s.d.. All statistics were obtained from 30 cells in five independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, two-tailed unpaired t-test.

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