Research Paper

Platycodin D Impairs the Oncogenic Phenotypes of Esophageal Squamous Cell Carcinoma Cells by Elevating microRNA-4319

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To probe the action of platycodin D in the oncogenic phenotypes of esophageal squamous cell carcinoma cells and its possible mechanism. Esophageal squamous cell carcinoma cell line EC9706 was exposed to different doses of platycodin D (2.5, 5 or 10 µmol/l). Untreated cells were adopted as control group. After indicated transfection, functional analyses were carried out. The proliferation, migration and invasion of EC9706 cells were dose-dependently decreased by platycodin D. Moreover, platycodin D treatment dose-dependently increased microRNA-4319 levels in cells. MicroRNA-4319 rise impaired EC9706 cell proliferation, invasiveness and migration. Besides that, the inhibition of microRNA-4319 abolished the action of platycodin D in EC9706 cells. Platycodin D could impair cell proliferation, migration and invasion abilities in esophageal squamous cell carcinoma through elevating microRNA-4319.

Key words: Platycodin D, esophageal squamous cell carcinoma, microRNA-4319, matrix metalloproteinase, platycodin D

Esophageal Squamous Cell Carcinoma (ESCC) is found to have increasing incidence rate and mortality rate in China in recent years[1]. ESCC is asymptomatic in the early stage; most people don't notice symptoms until after the cancer has spread. Currently, the surgery and/or radiotherapy remain the main therapies for ESCC, however, the survival time of ESCC patients is still dissatisfactory[2,3]. Thus, it is important to find effective therapeutic methods for ESCC.

Traditional Chinese Medicine (TCM) has anti-tumor, antioxidant stress, anti-inflammatory activity and so on, and is implicated in affecting ESCC development process[4,5]. Platycodin D (PD) is a triterpenoid saponin isolated and separated from the roots of *Platycodon grandiflorus*. Saponins are a large class of components with low side effects, low cost, easy to obtain and significant antitumor activity, which has become one of the most active and rapidly developing fields in Chinese medicine research, and is worthy of further research and development of anti-tumor new drugs. Recently, the anticancer action of PD has reported. Liu *et al.*[6] showed that PD could enhance cetuximab-induced metastasis and growth inhibition in colorectal cancer by mediating of Phosphoinositide 3-Kinase (PI3K)/Protein Kinase B (AKT) pathway inactivation. PD hampered the growth of gastric cancer by enhancing ubiquitination-induced c-Myc protein degradation[7]. However, there have been no reports on the relationship between PD and ESCC. MicroRNAs (miRNAs) can degrade or inhibit messenger Ribonucleic Acid (mRNA) translation post transcriptionally by binding with the mRNA, 3' Untranslated Regions (3' UTRs), thereby modulating diverse cellular functions[8,9]. A study exhibited that miR-4319 restrained ESCC growth *via* targeting NLRC5[10]. However, whether miR-4319 is implicated in modulating ESCC tumorigenesis and has the potential to be the therapeutic target remain unclear.

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Therefore, this study mainly probed whether PD and miR-4319 affected the oncogenic phenotypes of ESCC cells, and their relationship in ESCC tumorigenesis.

MATERIALS AND METHODS

Materials and reagents:

EC9706 cells, Dulbecco's Modified Eagle Medium (DMEM), and 10 % Fetal Bovine Serum (FBS) (American Type Culture Collection (ATCC), United States of America (USA)); PD (purity ≥98 %, Chengdu Yirui Biotechnology Co., Ltd.,); Trizol reagent, PrimeScript RT reagent, SYBR® Green reagent and Lipofectamine™ 3000 (Invitrogen, USA); miR-4319 mimic (miR-4319), inhibitor (anti-miR-4319), miR-NC and anti-miR-NC (RiboBio, Guangzhou, China); Matrigel (50 µg/ml, BD Biosciences, USA); Cell Counting Kit-8 (CCK-8), methanol, 1 % crystal violet, and paraformaldehyde (Beyotime, Shanghai, China); Transwell chamber (Corning Costar Corp., USA); Matrix Metalloproteinase (MMP)-2 (1:2000, ab92536), MMP-9 (1:2000, ab76003), Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) (1:1000, ab8245) primary antibodies, and secondary antibodies (Abcam, USA).

Experimental grouping:

EC9706 cells were cultured with DMEM plus 10 % FBS at 37° with 5 % Carbon dioxide (CO_2). For PD treatment, EC9706 cells were exposed to 2.5, 5 or 10 µmol/l PD for 24 h, named PD-L, PD-M and PD-H groups. Untreated cells were used as the control group. Lipofectamine™ 3000 was applied for transient transfection, EC9706 cells were transfected with anti-miR-4319 or anti-miR-NC, followed by 10 µmol/l PD treatment, named PD+anti-miR-NC and PD+anti-miR-4319 groups.

CCK-8 assay:

The each well of a 96-well plate was added with 2×10^3 EC9706 cells. Following assigned treatment and/or treatment, cells were reacted with CCK-8 10 µl reagent for 2 h, followed by examining the absorbance at 450 nm.

Colony formation assay:

The assigned EC9706 cells were cultured in six-well plate (500 cells/well) with DMEM for about (10-14) d. The medium was discarded, and the each well was added with 500 µl methanol to fix proliferating colonies for 20 min, followed by dyeing for 15 min with 400 µl crystal violet (1 %). Lastly, the colonies (≥50 cells) were photographed and counted.

Scratch assay:

Each group of EC9706 cells was inoculated into a 6-well plate (2×10^5 cells/well) and cultured in the incubator until per well was filled with cells. Then cells on the 6-well plate were scratched by a 20 µl sterile pipette tip and imaged under a microscope (0 h at the moment) after washing. Thereafter, cells were cultivated for 24 h in media without serum and then imaged. The distance was measured, and cell migration was assessed.

Transwell assay:

About 0.1 ml Matrigel diluent were added onto the plate surface of the upper Transwell chamber and allowed to solidify for 2 h. Then 1×10^5 assigned EC9706 cells were plated into the upper chamber with 500 µl complete medium on the lower chamber. 24 h later, invaded cells were observed and counted after dying with 0.1 % crystal violet.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR):

Total RNA was prepared by the assist of the TRIzol reagent, then complementary Deoxyribonucleic Acid (cDNA) were synthesized, followed by qRT-PCR amplification on a 7500 real-time PCR system. The relative of miR-4319 level was examined using by the comparative method.

Western blot:

EC9706 cells were reacted with 400 µl Radioimmunoprecipitation Assay (RIPA) lysate on ice for 30 min, followed by centrifuging 10 000 r/min for 10 min. The protein samples were denatured, loaded onto Sodium Dodecyl-Sulfate (SDS) gels, and then transferred onto Polyvinylidene Difluoride (PVDF) membranes. Thereafter, primary antibody diluents were used to incubate overnight with membranes at 4°, and following secondary antibody diluents at 37° for 2 h. The gray values were quantified by Quantity One software.

Statistical analyses:

The data were manifested as ($\bar{x}\pm s$). Student's t-test (two tailed) or one-way Analysis of Variance (ANOVA) analysis was adopted for comparisons between two or multiple groups. $p<0.05$ were meant to be statistically significant.

RESULTS AND DISCUSSION

PD treatment dose-dependently elevated EC9706 cell proliferation, evidenced by increased proliferation inhibitory rate and decreased colonies number (Table 1).

PD treatment dose-dependently elevated EC9706 cell migration and invasion, as well as MMP-2 and MMP-9 levels (fig. 1 and Table 2). As exhibited in Table 3, levels of miR-4319 were dose-dependently increased in PD-L, PD-M, and PD-H groups.

The introduction of miR-4319 mimic in EC9706 cells notably elevated miR-4319 levels, miR-4319 overexpression suppressed cell proliferation, demonstrating by increased proliferation inhibitory rate and declined colonies number (Table 4). miR-4319 elevation impaired EC9706 cell migration and invasion, as well as reduced MMP-2 and MMP-9 protein levels (fig. 2 and Table 5). miR-4319 inhibition reversed PD-mediated inhibition of EC9706 cell proliferation, migration and invasion, as well as the reduction of MMP-2 and MMP-9 protein levels (fig. 3 and Table 6).

Currently, chemotherapy and radiotherapy resistance are the main reasons for the reduced treatment efficacy of ESCC patients. TCM has the anticancer activity and can restrain the development process of ESCC by modulating gene expression[11]. At present, saponins isolated from TCM are found to have few toxic side effects and can improve tumors. Saponins can significantly increase the immune response of the body, prolong the immune cycle of vaccines as adjuvants, improve interferon regulatory factors, and swell the expression level of tumor necrosis factor and other immune-related genes, which promote the research prospect of Chinese medicine saponins as vaccine adjuvants and drug administration. PD is one of the widely used traditional herb with multiple pharmacological properties including antiviral, neuroprotective, anti-inflammatory, anti-aging, and anticancer activities[12-14]. PD repressed cell metastasis and proliferation in bladder cancer through XIST/miR-335 axis[15]. Cell growth and mobility could be impaired by PD in gallbladder cancer[16]. In addition, it was also found PD repressed the mobility of multiple myeloma cells *via* blocking the JAK2/STAT3 pathway[17]. Herein, results of this study showed that PD treatment dose-dependently repressed ESCC cell proliferative, migratory and invasive abilities. MMP-2 and MMP-9 are mobility-related markers, and was found to be elevated in ESCC[18]. In our work, we also showed that MMP-2 and MMP-9 protein levels were dose-dependently restrained by PD treatment, further suggesting the inhibition of PD on ESCC cell mobility.

TABLE 1: THE EFFECTS OF PD ON EC9706 CELL PROLIFERATION ($\bar{x}$±s, n=9)

Group	Proliferation inhibitory rate (%)	Colonies number
Control	0.00±0.00	86.46±6.53
PD-L	26.34±2.33*	70.04±5.75*
PD-M	43.68±4.04*#	51.91±4.87*#
PD-H	65.21±5.53*#&	36.82±3.26*#&
F	523.348	152.318
p	0.000	0.000

Note: Control *vs.* *p<0.05; PD-L *vs.* #p<0.05 and PD-M *vs.* &p<0.05

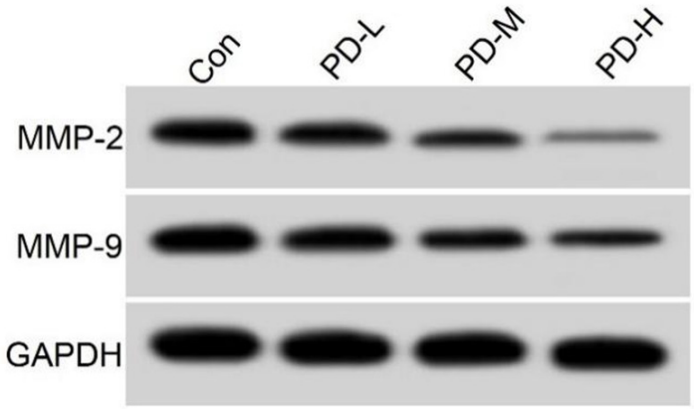

Fig. 1: PD treatment dose-dependently elevated EC9706 cell migration and invasion, as well as MMP-2 and MMP-9 levels

TABLE 2: ACTION OF PD ON EC9706 CELL MIGRATION AND INVASION

Group	Migration (%)	Invasion	MMP-2	MMP-9
Control	73.11±6.39	114.14±11.19	0.57±0.05	0.74±0.06
PD-L	57.02±5.08*	90.56±7.18*	0.43±0.04*	0.61±0.05*
PD-M	41.61±3.39*#	73.65±6.31*#	0.31±0.03*#	0.47±0.03*#
PD-H	27.65±2.57*#&	54.19±5.18*#&	0.19±0.02*#&	0.32±0.03*#&
F	163.309	95.846	176.667	149.013
p	0.000	0.000	0.000	0.000

Note: Control *vs.* *p<0.05; PD-L *vs.* #p<0.05 and PD-M *vs.* &p<0.05

TABLE 3: ACTION OF PD ON miR-4319 EXPRESSION IN EC9706 CELLS

Group	miR-4319
Control	1.00±0.00
PD-L	1.74±0.14*
PD-M	2.55±0.22*#
PD-H	3.32±0.23*#&
F	299.7
p	0.000

Note: Control *vs.* *p<0.05; PD-L *vs.* #p<0.05 and PD-M *vs.* &p<0.05

TABLE 4: EFFECTS OF miR-4319 ON EC9706 CELL PROLIFERATION

Group	miR-4319	Proliferation inhibitory rate (%)	Colonies
miR-NC	1.00±0.00	6.16±0.57	89.27±6.59
miR-4319	3.74±0.28*	56.74±4.83*	44.04±4.21*
t	29.357	31.200	17.352
p	0.000	0.000	0.000

Note: miR-NC *vs.* *p<0.05

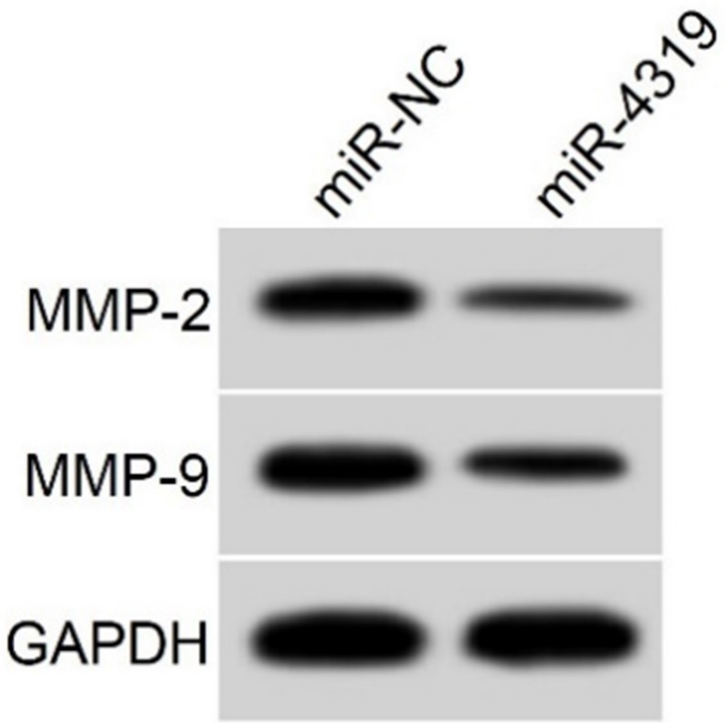

Fig. 2: miR-4319 elevation impaired EC9706 cell migration and invasion, as well as reduced MMP-2 and MMP-9 protein levels

TABLE 5: ACTION OF miR-4319 ON EC9706 CELL MIGRATION AND INVASION

Group	Migration (%)	Invasion	MMP-2	MMP-9
miR-NC	74.86±6.11	115.42±11.78	0.59±0.05	0.76±0.05
miR-4319	33.39±3.42*	62.34±5.12*	0.26±0.02*	0.39±0.04*
t	17.768	12.397	18.384	17.335
p	0.000	0.000	0.000	0.000

Note: miR-NC *vs.* *p<0.05

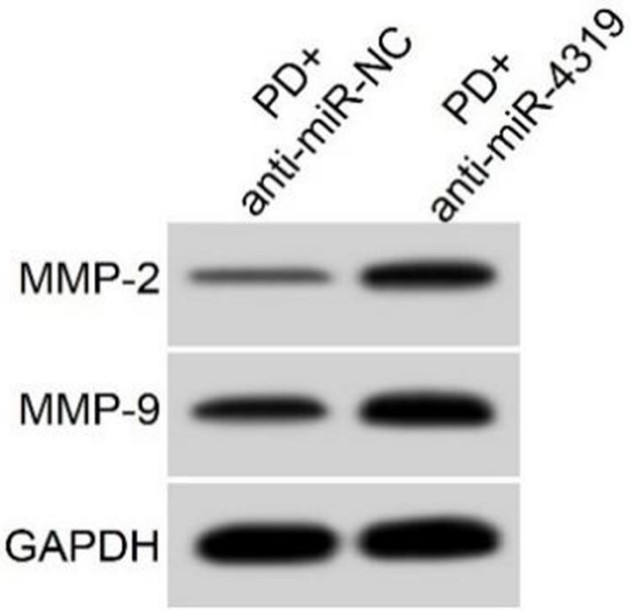

Fig. 3: miR-4319 inhibition abolished the effects of PD on EC9706 cells

TABLE 6: miR-4319 INHIBITION ABOLISHED THE ACTION OF PD ON EC9706 CELLS (x̄±s, n=9)

Group	miR-4319	Proliferation inhibitory rate (%)	Colonies	Migration (%)	Invasion	MMP-2	MMP-9
PD+anti-miR-NC	1.00±0.00	57.08±4.97	43.70±4.22	32.91±4.10	61.29±5.13	0.24±0.03	0.37±0.03
PD+anti-miR-4319	0.43±0.04*	12.85±1.22*	78.33±6.71*	66.78±5.59*	97.51±8.64*	0.48±0.04*	0.68±0.05*
t	42.75	25.928	13.106	14.657	10.814	14.400	15.949
p	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Note: PD+anti-miR-NC *vs.* *p<0.05

MiRNAs have been reported to be implicated in ESCC tumorigenesis *via* modulating the expression of target mRNA[19,20]. However, whether miRNA can serve as a potential target for PD in regulating ESCC remains unclear. miR-4319 is a functional miRNA, it was found to be decreased in colorectal cancer, the up-regulation of its expression could repress the growth of this cancer cells[21]. Thyroid cancer showed decreased miR-4319 levels, which led to cancer cell to migrate and proliferate[22]. In addition, miR-4319 performed antitumor activity in gastric cancer to impede cell growth[23]. In our work, we found PD treatment dose-dependently resulted in the increase of miR-4319 content in ESCC cells. The overexpression miR-4319 was proved to suppress ESCC cell to migrate, invade and proliferate. As expected, MMP-2 and MMP-9 levels were also declined after miR-4319 elevation. Thereafter, we probed whether miR-4319 was implicated in the action of PD in ESCC cells. The result displayed that miR-4319 deficiency abolished the anticancer action of PD on ESCC cells, evidenced by enhanced migration, invasion and proliferation of ESCC cells in high miR-4319 group under PD treatment.

In summary, PD treatment restrained ESCC cell invasion, migration and proliferation. Its mechanism of action might be linked with the promotion of miR-4319 expression, which provide new directions for developing therapeutic drugs for ESCC.

Conflict of interests:

The authors declared no conflict of interests.

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