Research Paper

Effect of Plantamajoside on Malignant Behaviors of MHCC97H Cells by Mediating microRNA-3178 Expression

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The objective of this study is to explore the effect of plantamajoside on malignant properties of MHCC97H cells and its possible mediating molecular mechanisms. Effects of high, median, and low concentrations of plantamajoside on MHCC97H cell malignant behaviors were assessed. Quantitative reverse transcriptase polymerase chain reaction analysis detected microRNA-3178 expression in tissue samples. Western blotting detected E-cadherin and N-cadherin protein levels. Plantamajoside led to a remarkable reduction in the proliferative, migrating and invasive capabilities of MHCC97H cells, which was accompanied with a diminution in N-cadherin protein levels as well as an augmentation in E-cadherin protein level and microRNA-3178 expression. Reduction of microRNA-3178 was noted in hepatocellular carcinoma samples *vs*. para-carcinoma tissues. Furthermore, microRNA-3178 overexpression demonstrated an ability to inhibit the effects on the invasion, metastasis, and proliferation of MHCC97H cells. Amusingly, microRNA-3178 silencing attenuated the plantamajoside-mediated inhibitory impact on malignant behaviours of MHCC97H cells. Plantamajoside treatment resulted in an upregulation of microRNA-3178, thus repressing MHCC97H cell invasion, metastasis, and proliferation.

Key words: MHCC97H cells, plantamajoside, microRNA-3178, *Plantago asiatica*, hepatocellular carcinoma

Hepatocellular Carcinoma (HCC) is a complex malignant tumor with lethality, ranking 6th in global mortality rates[1]. Multiple factors contribute to the emergence of this disease, including fatty liver caused cirrhosis, carcinogenic toxins, viral infections, and miscellaneous genetic factors[2,3]. Interventions for HCC include surgical resection, transarterial chemoembolization, local ablation, sorafenib therapy, and liver transplantation[4]. Despite these robust therapeutic interventions, survival in patients with HCC remains sluggish[5,6]. Therefore, more effective drugs with minimal toxicity should be developed to improve overall survival in HCC patients.

Plantago asiatica L., a plant of the family Plantaginaceae, has the effects of clearing heat and drenching, expelling phlegm, diuretic and cooling blood[7]. *Plantago* contains a variety of active substances, among which Plantamajoside (PMS) is a representative component for evaluating the quality of *Plantago* in the 2020 edition of the Chinese Pharmacopoeia[8]. As confirmed by studies, PMS has anti-inflammatory[9], anti-oxidant[10], immunomodulatory[11], and anti-tumor properties[12]. For instance, improvement of Adenosine Monophosphate-activated Protein Kinase (AMPK)/Nuclear factor erythroid 2-related factor 2 (Nrf2) by PMS regulated hepatic lipid metabolism and immune dysregulation in Nonalcoholic Fatty Liver Disease (NAFLD) rats[11]. Inflammatory responses in Lipopolysaccharide (LPS) stimulated human gingival fibroblasts were attenuated by PMS through repression of the Phosphoinositide-3-Kinase/Protein Kinase B (PI3K/Akt) pathway[13]. PMS represses cancer cell metastasis and growth in manifold tumors, such as melanoma[14], cervical cancer[15], and HCC[16]. At present, the molecular mechanisms related to PMS-mediated inhibition of HCC have not been well elucidated.

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MicroRNAs (miRNAs), a large family of single-stranded Ribonuclic Acid (RNA) molecules (18-25 nucleotides), play a critical role as post-transcriptional regulators[17]. Regarding the regulatory role of miRNAs in various gene expression levels, dysfunction or dysregulation of the expression of these molecules can lead to various diseases, including various types of cancer[18]. As observed across miscellaneous studies, miRNA dysregulation has been recognized as a causative mechanism behind HCC tumorigenesis[19]. PMS has been reported to decrease S100 calcium-binding protein A16 (S100A16) expression by elevating miR-711 expression, inhibiting bladder cancer cell metastasis and proliferation[20] and whether PMS affects the malignant behavior of HCC cells by regulating miRNA levels has not been well elucidated. However, a tumor-suppressive miRNA, miR-3178 has been reported in other cancers. Dysregulation of miR-3178 has been associated with the reversal of gemcitabine resistance in pancreatic cancer[21]. miR-3178 also refers to the carcinogenesis of HCC[22], breast cancer[23] and gastric cancer[24]. Currently, it is unclear whether PMS affects HCC cell malignancy by regulating the levels of miR-3178.

Thus in this study, whether PMS inhibits the malignant behaviour of HCC cells by mediating miR-3178 expression was addressed.

MATERIALS AND METHODS

Cell culture, experimental grouping and transfection:

MHCC97H cells (Honsun Biological Technology Co., Ltd.) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, USA) supplemented with 10 % Fetal Bovine Serum (FBS).

MHCC97H cells were incubated in DMEM medium containing High (H) (200 µg/ml), Low (L) (50 µg/ml), and Moderate (M) (100 µg/ml) concentrations of PMS (Shanghai Yuanye Biological Technology Co., Ltd.) and was categorized as PMS-L, PMS-M and PMS-H groups, respectively.

For transient transfection of miR-Negative Control (NC), miR-3178 mimics, anti-miR-NC, anti-miR-3178 (Ribobio, Guangzhou, China) and Lipofectamine™ 3000 (Invitrogen) was utilized. After successful transfection, the cells were cultured in DMEM medium containing PMS (200 µg/ml) for 24 h and labelled as PMS+anti-miR-NC and PMS+anti-miR-3178 groups, respectively.

Cell proliferation analysis:

After incubation of MHCC97H cells (3×10^3 cells/well) for 24 h, 10 µl of the Cell Counting Kit-8 (CCK-8) solution (Solarbio, Beijing, China) was added. By applying a plate reader (Tecan, Group Ltd., Switzerland), we tested the Optical Density (OD) at 450 nm in each well. Calculation of cell proliferation inhibition rate was performed by calculating, (OD control-OD Experiment)/(OD control-OD blank)×100 %.

For clone formation assay, MHCC97H cells (500 cells/well) were inoculated and continued to be cultured until visible cell clones. Fixation of cells was performed by adding 500 µl of methanol (Solarbio Life Science, China). After 20 min, 1 % crystal violet staining solution staining (Solarbio) was added. Counting of the number of cell clones (≥50 cells as 1 clone) was carried out under the microscope.

Wound healing assay:

Inoculation of approximately 1×10^5 MHCC97H cells/well into 6-well plates was done. When the cells were grown fully, wounding was formed by scribing a line across the cell monolayer with a sterile pipette tip (200 µl). Imaging was performed under a microscope at 0 and 24 h. ImageJ software was applied to detect the migration distance. Calculation of the healing rate was done using the formula, (Width 0 h-width 24 h)/(Width 0 h×100 %).

Transwell assay for cell invasion:

Diluted Matrigel (300 µg/ml and 45 µl/well) was added to the upper chamber (Solarbio). MHCC97H cells (1×10^5 cells/well) were added to the upper chamber and medium containing 10 % FBS (500 µl) was added to the lower chamber subsequently. Following 24 h of incubation, fixation was carried out with paraformaldehyde (Solarbio). After 10 min of staining with 0.1 % crystal violet the cells were observed under the microscope.

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

Isolation of total RNA was accomplished

using Total RNA Isolation (TRIzol) reagent (Thermo, USA). Using the miScript® II RT kit (Applied Biosystems, USA), complementary Deoxyribonucleic Acid (cDNA) was synthesized following the manufacturer's recommendations. miR-3178 expression was analyzed using qRT-PCR using the TaqMan miRNA assay (Applied Biosystems). Small nuclear RNA (snRNA) U6 was used for normalization and $2^{-\Delta\Delta Ct}$ method was applied for the determination of the relative expression levels.

Western blotting:

Lysis of MHCC97H cells was performed with Radio-Immunoprecipitation Assay (RIPA) buffer (Beyotime, Shanghai, China). Following the detection of protein concentration, 40 µg of protein samples were subjected to Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The membranes were incubated with the primary antibodies against Epithelial (E)-cadherin (1:1000, Abcam, Massachusetts, USA), Neural (N)-cadherin (1:1000, Abcam), and Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) (1:3000, Abcam). Protein expression was detected by chemiluminescence using SuperSignal™ West Pico PLUS (Thermo Fisher Scientific, Inc.). Analysis of the grey values of each band was performed applying ImageJ software.

Statistical analysis:

The Graph Pad Prism 8 program (Graph Pad, Inc., San Diego, CA, USA) was used for the statistical analysis. The data was submitted to normalization analysis (Shapiro-Wilk test). Statistical significance was determined by student's t test. Comparisons were made in multiple comparisons using one-way Analysis of Variance (ANOVA) procedures. Data were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

In contrast to the control group, PMS resulted in increased proliferation inhibition as well as reduced number of clone formation in MHCC97H cells in a dose-dependent manner (Table 1).

Further experiments yielded that PMS caused a dose-dependent decrease in wound healing rate and invasive capacity of MHCC97H cells compared to the control group (Table 2). Simultaneously, PMS caused lowering of N-cadherin protein levels as well as an increase of E-cadherin protein levels, and these changes occurred in a dose-dependent pattern (fig. 1).

In comparison to para-cancerous tissues, miR-3178 was reduced markedly in HCC samples (Table 3). Comparing with the control group, PMS provoked a remarkable elevation of miR-3178 expression in MHCC97H cells in a dose-dependent pattern (Table 4).

The introduction of miR-3178 expression resulted in a prominent degradation of the proliferative, migratory, and invasive capacities of MHCC97H cells when compared to the miR-NC group (Table 5). A strongly elevated levels of E-cadherin protein and reduced levels of N-cadherin protein levels were observed in miR-3178 mimic-transfected MHCC97H cells (fig. 2).

MHCC97H cells in the PMS+anti-miR-NC group showed increased proliferative, migratory, and invasive potentials by comparison with those in the PMS+anti-miR-NC group (Table 6) along with diminished levels of E-cadherin protein and augmented levels of N-cadherin protein (fig. 3).

TABLE 1: PMS REPRESSED MHCC97H CELL PROLIFERATION (n=9)

Group	Inhibitory rate (%)	Number of cell clone formation (n)
Control	0.00±0.00	96.82±6.25
PMS-L	25.54±2.11*	79.07±5.61*
PMS-M	45.16±4.21*#	61.25±5.01*#
PMS-H	64.17±5.59*#&	44.15±3.92*#&
F	508.089	167.128
p	0.000	0.000

Note: &p<0.05, #p<0.05 and *p<0.05 with respect to PMS-M, PMS-L and control group respectively

TABLE 2: PMS RESTRAINED MHCC97H CELL METASTASIS AND INVASION (n=9)

Group	Wound healing rate (%)	Number of cell invaded (n)
Control	72.14±5.64	125.47±11.24
PMS-L	55.11±4.65*	91.39±8.03*
PMS-M	39.35±3.71*#	72.57±6.08*#
PMS-H	23.16±2.26*#&	54.27±5.17*#&
F	219.720	130.794
p	0.000	0.000

Note: &p<0.05, #p<0.05 and *p<0.05 with respect to PMS-M, PMS-L and control group respectively

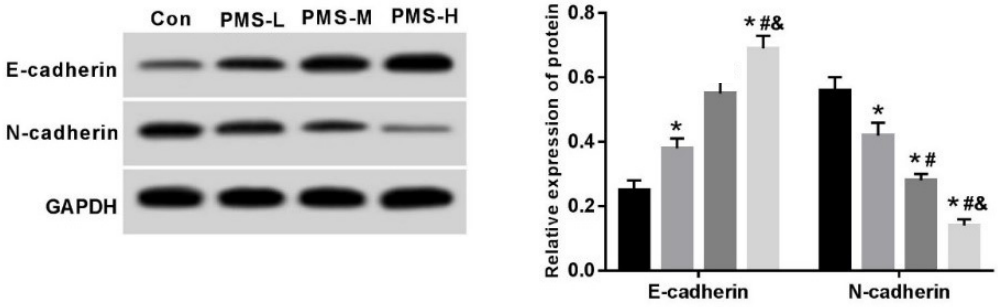

Fig. 1: Analysis of N-cadherin and E-cadherin protein levels in MHCC97H cells of PMS-L, PMS-M, and PMS-H groups (n=9)
Note: &p<0.05, #p<0.05, *p<0.05 with respect to PMS-M, PMS-L and control group ( ): Control; ( ): PMS-L; ( ): PMS-M and ( ): PMS-H

TABLE 3: LEVELS OF miR-3178 IN HCC SAMPLES (n=41)

Group	miR-3178
Para-cancerous tissues	1.00±0.12
HCC tissues	0.37±0.04*
t	31.891
p	0.000

Note: *p<0.05, in comparison with para-cancerous tissues

TABLE 4: PMS ELEVATED miR-3178 LEVELS IN MHCC97H CELLS (n=9)

Group	miR-3178
Control	1.00±0.00
PMS-L	1.72±0.13*
PMS-M	2.55±0.22*#
PMS-H	3.11±0.26*#&
F	232.677
p	0.000

Note: &p<0.05, #p<0.05 and *p<0.05 with respect to PMS-M, PMS-L and control group respectively

TABLE 5: miR-3178 OVEREXPRESSION REPRESSED MHCC97H CELL INVASION, METASTASIS AND PROLIFERATION (n=9)

Group	miR-3178	Inhibitory rate (%)	Number of cell clone formation (n)	Wound healing rate (%)	Number of invaded cells (n)
miR-NC	1.00±0.00	5.88±0.48	99.34±7.48	73.15±6.35	127.51±12.29
miR-3178	3.24±0.27*	50.16±4.75*	52.01±4.91*	32.61±3.12*	60.68±5.45*
t	24.889	27.825	15.869	17.190	14.913
p	0.000	0.000	0.000	0.000	0.000

Note: *p<0.05, with respect to miR-NC

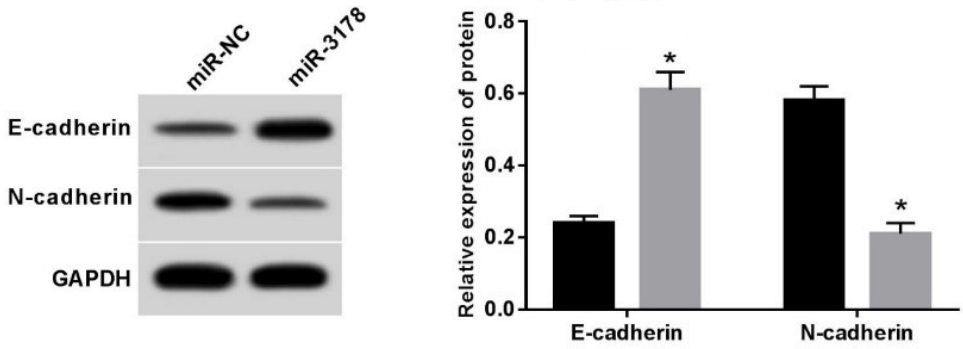

Fig. 2: Elevated levels of N-cadherin and E-cadherin in MHCC97H cells (n=9)
Note: ( ): miR-NC and ( ): miR-3178

TABLE 6: REPRESSION OF miR-3178 EXPRESSION RETROGRADED WITH PMS-MEDIATED IMPACTS ON MHCC97H CELL INVASION, METASTASIS AND PROLIFERATION (n=9)

Group	miR-3178	Inhibitory rate (%)	Number of cell clone formation (n)	Wound healing rate (%)	Number of cell invaded (n)
PMS+anti-miR-NC	1.00±0.00	66.83±4.84	43.28±3.64	21.97±2.21	51.78±4.72
PMS+anti-miR-3178	0.46±0.04*	27.15±2.63*	82.05±5.62*	61.26±5.03*	106.64±11.52*
t	40.500	21.611	17.371	21.454	13.220
p	0.000	0.000	0.000	0.000	0.000

Note: *p<0.05, with respect to PMS+anti-miR-NC

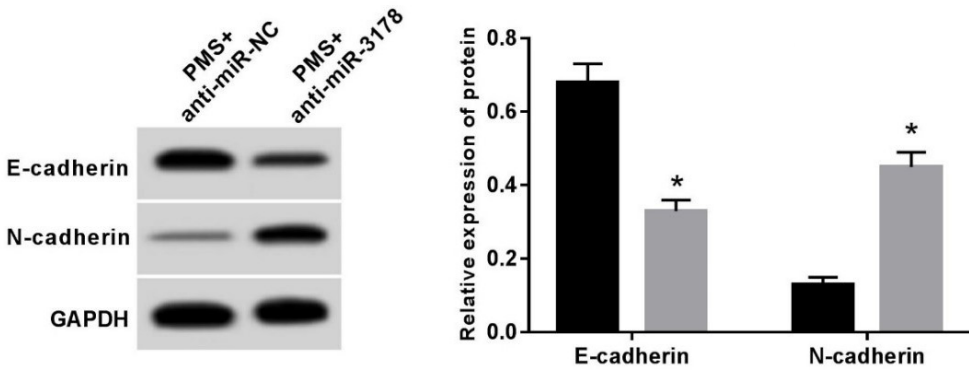

Fig. 3: Lowered levels of N-cadherin and E-cadherin in MHCC97H cells (n=9)
Note: ( ): PMS+anti-miR-NC and ( ): PMS+-miRNC

Early symptoms of HCC are relatively insidious, leading to poor prognosis, and there are no effective drugs and treatments to control the metastasis of HCC cells. Prior investigations have shown that some traditional Chinese medicines can facilitate HCC cell apoptosis and prohibit the metastasis and growth of HCC cells[25,26]; aberrantly expressed miRNAs were identified in HCC. Considerable studies have been conducted to confirm that miRNAs can modulate the biological behaviours of HCC cells and may also work as potential targets for HCC treatment[27,28]. However, whether miRNAs can be used as potential targets for drug treatment of HCC needs to be elucidated.

A previous study revealed that PMS inhibited HCC cell proliferation and metastasis[16]. As a result of inhibiting Akt/Glycogen Synthase Kinase-3 Beta (GSK3β) signaling, PMS fostered metformin-induced autophagy and apoptosis in HCC cells[29]. Similarly, PMS was shown to restrain the growth and metastasis of malignant melanoma[14]. Lowering of Matrix Metalloproteinase (MMP)-9/2 activities by PMS inhibited lung metastasis and tumor growth in breast cancer[30]. Inhibition of Akt and p38 Mitogen-Activated Protein Kinases (MAPK) phosphorylation by PMS regulated apoptosis and proliferation in lung cancer[31]. Here, PMS raised the rate of proliferative inhibition of MHCC97H cells and reduced the clone-forming capability in a concentration-dependent pattern, suggesting that PMS could regulate the proliferative capability of MHCC97H cells. At Epithelial-Mesenchymal Transition (EMT) process, N-cadherin and E-cadherin belong to a marker of mesenchymal cells and epithelial cells, respectively. Dysregulation of N-cadherin and E-cadherin can drive the EMT transition, thereby encouraging cell metastasis[32]. Findings of the present study were indicative of the fact that PMS treatment brought about a decrease in MHCC97H cell invasion and metastasis in a concentration-dependent pattern, accompanied by lowering of N-cadherin protein levels and up-regulation of E-cadherin protein levels, suggesting that PMS suppressed the invading and migrating abilities of HCC cells.

Downregulation of miR-3178 contributed to gastric cancer cell invasion[24]. By targeting Tumor necrosis factor Receptor Associated Factor 3 (TRAF3), miR-3178 suppressed the gastric cancer advancement[33], with low miR-3178 levels in breast cancer[34,35]. According to the results of current investigation, miR-3178 levels in HCC tissues were lower. With increase in PMS concentrations, miR-3178 expression in MHCC97H cells also increased progressively, suggesting that PMS might have an anti-cancer action by upregulating miR-3178 expression in HCC. Meanwhile, we found that miR-3178 overexpression restricted MHCC97H cell invasion, metastasis, and proliferation, whereas suppression of miR-3178 attenuated the inhibiting effects mediated by PMS, suggesting that PMS may regulate HCC cell growth and metastasis by increasing miR-3178 expression.

Collectively, PMS might restrict malignant behaviours of MHCC97H cells by upregulating miR-3178. This study provides a new direction regarding the therapeutic drug development for HCC and lays down the experimental foundation for further revealing about the mechanism of action of PMS against HCC. However, the underlying molecular mechanisms of PMS-mediated inhibitory effects on HCC still need to be further investigated.

Conflict of interests:

The authors declared no conflict of interests.

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