EFFECTS OF URSOLIC ACID ON THE PROLIFERATION, ADHESION AND INVASION OF A549 LUNG ADENOCARCINOMA CELLS AND THE EXPRESSION OF GLYCOSYLTRANSFERASE GENE B3GNT8

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ABSTRACT

Objective: *To investigate the effects of ursolic acid on the proliferation, adhesion and invasion of A549 lung adenocarcinoma cells and the expression of glycosyltransferase gene β3GnT8.*

Methods: *The effects of ursolic acid at different concentrations (5μmol/L,10μmol/L, 20μmol/L, 30μmol/L, 40μmol/L, 50μmol/L) on the proliferation of A549 cells were detected by MTT assay; adhesion test and Transwell invasion test were used to detect the effects of low concentration (10μmol/L) ursolic acid and high concentration (40μmol/L) ursolic acid on the adhesion and invasion ability of A549 cells; the expression of β3GnT8 gene at mRNA level was detected by fluorescence quantitative PCR, and the expressions of CD147 and MMP-2 at mRNA and protein levels were further detected.*

Results: *With the increase of ursolic acid concentration, the ability of proliferation, adhesion and invasion of A549 cells was lower than that of the control group (P<0.05), showing a concentration dependence; the expression of β3GnT8 at mRNA level decreased (P<0.05), and the expression of CD147 and MMP-2 at mRNA and protein level also decreased(P<0.05).*

Conclusion: *Ursolic acid could inhibit the proliferation, adhesion and invasion of A549 lung adenocarcinoma cells, and its mechanism may be related to the down-regulation of β3GnT8 gene expression.*

Keywords: *Ursolic acid, β3GnT8, proliferation, adhesion, invasion, A549 lung adenocarcinoma cells.*

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Background

Lung cancer is a common malignant tumor in clinic practices. In recent years, its morbidity and mortality have increased significantly(1-4). Preventing its invasion and metastasis has become a hot spot in current research. Ursolic acid (UA) is a pentacyclic triterpenoid compound. It exists in the roots, stems, leaves, and fruits of various plants, and it is an effective component of many Chinese herbal medicines. It has extensive biological functions(5-8), including anti-tumor effect, lowering blood lipid, lowering blood sugar, anti-inflammation, liver protection and lowering blood pressure, among which the Anti-tumor effect is its main pharmacological action.

During the invasion and metastasis of tumor cells, the activity of one or more glycosyltransferases changes, which leads to the abnormal structure of glycoprotein sugar chain, and further affect the biological behavior of the tumor, such as the proliferation, adhesion and infiltration of tumor cells. In this experiment, the effects of ursolic acid on the proliferation, adhesion and invasion of A549 lung adenocarcinoma cells and the expression of glycosyltransferase gene β3GnT8 were studied to explore the anti-tumor mechanism of UA.

Materials and methods

Materials

A549 lung adenocarcinoma cells were purchased from the cell bank of Shanghai Academy of Life Sciences, Chinese Academy of Sciences; cell culture dish, cell culture bottle and Transwell chamber were purchased from Corning company; DMEM medium, fetal bovine serum and trypsin were purchased from GIBCO Company; MTT cell proliferation kit was purchased from Sigma company; Trizol was purchased from Ambion company; real-time fluorescence quantitative PCR detection kit and reverse transcription kit were purchased from VAZYME Company; the primers used in the experiment were synthesized by Beijing Qingke Biotechnology Co., Ltd.; rabbit polyclonal antibody GAPDH was purchased from Hangzhou Goodhere Biological Co., Ltd.; both CD147 primary antibody and MMP-2 primary antibody were purchased from Wuhan Proteintech Group, Inc; HRP labeled sheep anti-rabbit secondary antibody was purchased from Wuhan BOSTER Biological Technology Co., Ltd.; the developing and fixing kit was purchased from Tianjin Hanzhong Photographic Material Factory; the electroluminescent ECL substrate solution was purchased from Nun Company under Thermo; 6-well plates and 96-well plates were all purchased from Nunc Company under Thermo; BCA protein quantitative kit was purchased from Beyotime Biotechnology Co., Ltd.

Methods

A549 cell culture and passage

A549 cells were taken out of liquid nitrogen, resuscitated, and suspended in a complete medium containing 10% fetal bovine serum. After that, they were inoculated into a culture bottle, gently blown and mixed, and cultured at 37°C and 5%CO_2 saturated humidity. When the density of cells reached 80%, the cells were digested with 0.25% pancreatin and passaged at a ratio of 1:3.

MTT assay of cell proliferation ability

A549 cells in logarithmic growth phase and in good growth state were inoculated in 96-well plates according to 5x103 cells/well, and 100 mL of culture solution was added to each well, which was cultured overnight in 5%CO_2 incubator at 37°C. The cells were treated according to the following groups: A549 cells (control group); A549 cells +5μmol/L ursolic acid group (5μmol/L group); A549 cells +10μmol/L ursolic acid group (10μmol/L group); A549 cells +20μmol/L ursolic acid group (20μmol/L group);A549 cells +30μmol/L ursolic acid group (30μmol/L group); A549 cells +40μmol/L ursolic acid group (40μmol/L group); A549 cells +50μmol/L ursolic acid group (50μmol/L group), and the action time of these groups were 24h.

Then, 10ul MTT was added to each well, which was then cultured at 37°C for 4h. After that, the culture medium was sucked out, and 150μl DMSO was added. After shaking for 10min, the absorbance value OD 568 of each well was measured by microplate reader. Proliferation rate = OD of experimental group/OD of control group × 100%.

Detection of cell adhesion ability

The 96-well plates were coated with 100μg/ml fibronectin. A549 cells in logarithmic growth phase and in good growth state were inoculated into 6-well plates for cell culture with $2×10^5$ cells per well. The cells were treated and divided into the following groups: A549 cells (control group), A549 cells +10μmol/L ursolic acid group (low concentration group), A549 cells +40μmol/L ursolic acid group (high concentration group) with action time of 24 h. The cell concentration was adjusted to $5×10^5$ cells/ml. 100μl cell suspension was added to each hole of the coated cell culture plate. Then, the plate was centrifuged at low speed for 2 min to make the cells settle at the bottom of the plate. After that, the plate was cultured in a 5% CO_2 incubator at 37°C for 30min. After sucking to clear the non-adhered cells, the plates were washed with PBS twice. A group of normal cells were set as the total cell group without sucking to clear the cells. 100μl culture medium was added to each well, and pictures were taken with a high-power mirror (×200). 10μl MTT was added to each well. After that, the cells were cultured at 37°C for 4h. The absorbance value OD568 of each hole was measured by microplate reader. Adhesion percentage = absorbance value of each group/ absorbance value of total groups×100%. The experiment was repeated three times.

Detection of cell invasion ability

The cells were inoculated into the 6-well plates with $2×10^5$ cells per well, and were cultured for 24h, the treatment and group can be seen in part 2.2.3. Precooled DMEM culture medium was added into 24-well plate, which was then put it into transwell

chamber. 100μL1mg/mL Matrigel was vertically added into the center of the bottom of the upper chamber of transwell chamber, incubated at 37°C for 4-5 h to make it gel. After that, 200μL cell suspension of each group was added into the upper chamber of transwell respectively. The mixture was put in a 5% CO_2 incubator at 37°C.

After culturing for 24h, the transwell was taken out, and carefully cleaned with PBS, and cleaned the cells on the chamber with a cotton swab. The cells were fixed with 70% ice ethanol solution for 1h, and then stained with 0.5% crystal violet dye solution. They were observed under a microscope (×200), and photos were taken. The number of transmembrane cells was counted, and the invasion ability of cells was expressed with the number of transmembrane cells. The experiment was repeated three times.

Real-time fluorescence quantitative PCR detection of the mRNA expressions of β3GnT8, CD147 and MMP-2 in three groups of cells

Three groups of cells (control group, low concentration group and high concentration group) in logarithmic growth phase were collected respectively. Total RNA was extracted according to the operating manual of Trizol Reagent (Invitrogen company), and OD260 and OD280 were determined by spectrophotometry to calculate the purity and concentration of RNA.

The ratio of OD260/OD280 was between 1.8 and 2.0, meeting the experimental requirements. 5 μg total RNA was taken for reverse transcription reaction, and then PCR amplification was carried out. GAPDH was used as an internal reference.

The upstream primer of β3GnT8 was 5'-GCTGCCCTTTGCTTACTG-3', and its downstream primer was 5'-GCCCTGGTTCTGACTTGA -3; the upstream primer of MMP2 was 5'-TATGA-CAGCTGCACCACTGA -3', and its downstream primer is 5'-TCATCGTAGTTGGCTGTGGT-3'; the upstream primer of CD147 was 5'-CTCCTG-CGTCTTCCTCCCCG -3', and its downstream primer was 5'-ATGGCCGTCTCCCCCTCGTT -3'.

The expressions of β3GnT8, CD147 and MMP-2 were analyzed by $2^{-\triangle\triangle Ct}$. The experiment was repeated three times, and the mean value was taken.

Western Blot detection of the protein expressions of CD147 and MMP-2 in three groups of cells

Total protein was extracted from monolayer adherent cells. Protein was quantified by BCA, and linear regression equation was calculated according to standard protein concentration and corresponding OD value. The protein concentration of samples was calculated by regression equation according to OD value of protein samples. The protein was denatured in boiling water bath for 10min, and then electrophoresed by SDS-PAGE. The PVDF membrane was soaked in TBST (sealing solution) containing 5% skimmed milk powder for 2h. Then, it was diluted with primary antibody and sealed overnight. The dilution ratio was as follows: 1:1000 for GAPDH, 1:200 for CD147, 1:500 for MMP-2. After incubation with secondary antibody in shaking table for 2h, the ECL system was used for immunoblotting. The film was scanned, and the gray value of the film was analyzed by BandScan.

Statistical analysis

The data were processed by SPSS20.0 statistical software. The measurement data were expressed as mean±standard deviation ($\bar{x}\pm s$). The comparison between sample means was analyzed by one-way ANOVA, and the pairwise comparison between groups was tested by LSD-t. In case of no normal distribution, the nonparametric test would be carried out; $P<0.05$ indicated a statistically significant difference.

Results

Effect of ursolic acid with different concentrations on proliferation of A549 cells

Compared with the control group, ursolic acid with different concentrations could significantly inhibit the proliferation of A549 cells, and the difference was statistically significant ($P<0.05$). Compared with the control group and the previous group, the cell proliferation rate of each group decreased significantly ($P<0.05$). See Table 1.

Group	Cell proliferation rate (%)
Control group	100
5μmol/L group	93.89±2.09①
10μmol/L group	84.22±2.91②
20μmol/L group	67.88±1.91③
30μmol/L group	57.80±2.44④
40μmol/L group	46.65±1.20⑤
50μmol/L group	43.19±1.37⑥

Table 1: Effects of ursolic acid with different concentrations on A549 cell proliferation (n=3, $\bar{x}\pm s$).

Note: Compared with the control group: ① P<0.05; Compared with the 5μmol/L group: ② p<0.05; Compared with the 10μmol/L group: ③ p<0.01; Compared with the 20μmol/L group: ④ p<0.01; Compared with the 30μmol/L group: ⑤ p<0.01; Compared with 40μmol/L group: ⑥ p<0.05.

Effect of ursolic acid on cell adhesion and invasion

Compared with the control group (A549 group), the fibronectin adhesion percentage and invasion number in the low concentration group decreased significantly (P<0.05).

Compared with the control group and the low concentration group, the fibronectin adhesion percentage and invasion number in the high concentration group decreased significantly (P<0.05). See Figure 1, Figure 2 and Table 2.

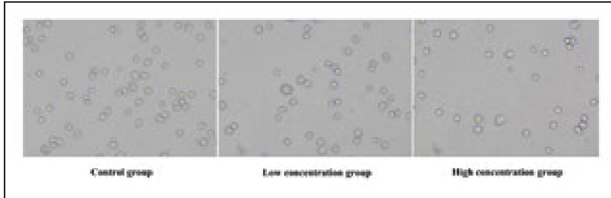

Figure 1: The adhesion of cells in each group to fibronectin observed under microscope (×200).

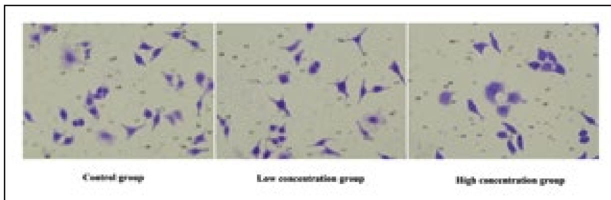

Figure 2: The invasion of cells in each group observed under microscope (×200).

Group	Adhesion percentage (%)	Number of invasion (cell)
Control group	50.21±3.27	36.15±2.16
Low concentration group	37.08±2.65$^{\triangle}$	26.11±2.06$^{\triangle}$
High concentration group	24.32±2.31$^{\star\blacktriangle}$	20.35±3.07$^{\star\blacktriangle}$

Table 2: Effect of ursolic acid on cell adhesion and invasion (n=3, $\bar{x}$±s).
Note: Compared with the control group: $^{\triangle}$*P<0.05; Compared with the control group:* **P<0.05; Compared with the low concentration group,* $^{\blacktriangle}$*P<0.05.*

Real-time fluorescence quantitative PCR detection of the mRNA expressions of β3GnT8, CD147 and MMP-2

The mRNA expression of β3GnT8 in the low concentration group was significantly lower than that in the control group, and the mRNA expression of β3GnT8 in the high concentration group was significantly lower than that in the control group and the low concentration group (p<0.05).

The mRNA expressions of CD147 and MMP-2 in the low concentration group were significantly lower than those in the control group, and the mRNA expressions of CD147 and MMP-2 in the high concentration group were significantly lower than those in the control group and the low concentration group (p<0.05). See Table 3.

Group	β3GnT8	CD147	MMP-2
Control group	1.000	1.000	1.000
Low concentration group	0.516±0.065$^{\triangle}$	0.361±0.046$^{\triangle}$	0.345±0.025$^{\triangle}$
High concentration group	0.261±0.028$^{\star\blacktriangle}$	0.119±0.027$^{\star\blacktriangle}$	0.117±0.016$^{\star\blacktriangle}$

Table 3: Relative mRNA expressions of β3GnT8, CD147 and MMP-2 in different groups (n=3, $\bar{x}$±s).
Note: Compared with the control group: $^{\triangle}$*P<0.05; Compared with the control group:* **P<0.05; Compared with the low concentration group,* $^{\blacktriangle}$*P<0.05.*

Western Blot detection of the protein expressions of CD147 and MMP-2

The protein was extracted from cells of the control group, low concentration group, and high concentration group, and the expressions of CD147 and MMP-2 were detected by Western blot. Compared with the control group, the expressions of CD147 and MMP-2 in the low concentration group significantly decreased, while those in the high concentration group were significantly lower than those in the low concentration group. See Figure 3.

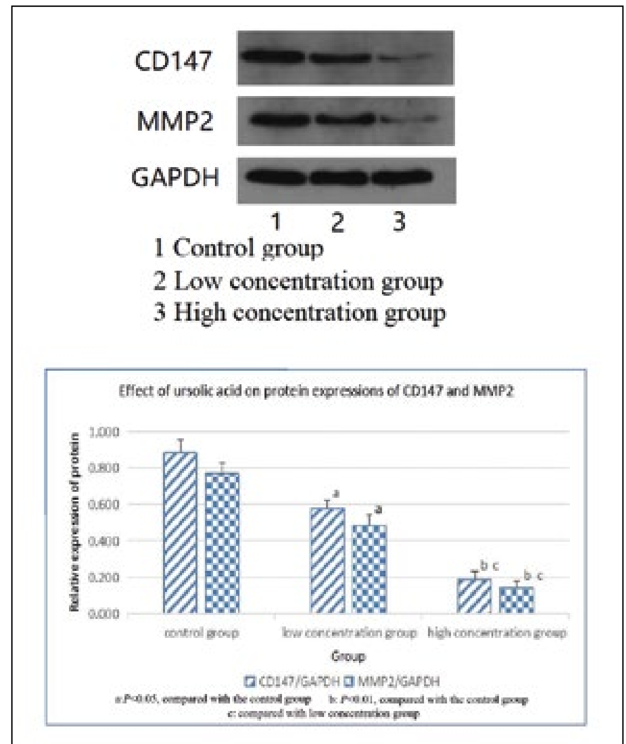

Figure 3: Effects of ursolic acid with different concentrations on protein expressions of MMP2 and CD147.

Discussion

Recent studies have found that ursolic acid can inhibit the growth of various tumor cells(9-12) and has an anti-tumor effect. It has been reported that ursolic acid can inhibit the proliferation of human liver

cancer cells and induce apoptosis of liver cancer cells in a dose-dependent manner[13]; ursolic acid can inhibit the migration, proliferation and invasion of lung cancer A549 cells by down-regulating miRNA-21 on PTEN/PI3K/AKT pathway[14]; ursolic acid can inhibit the proliferation of ovarian cancer cells by down-regulating the expression of adenosine triphosphate-binding cassette superfamily G member 2 (ABCG2)[15]. However, there are few reports on the effect of ursolic acid on adhesion and invasion of lung cancer cells and its mechanism from the perspective of glycosyltransferase.

In the process of growth, differentiation, proliferation, infiltration and metastasis of malignant tumor cells, glycosylation modification on the cell surface often changes significantly. This process needs glycosyltransferase to participate in glycosylation reaction to catalyze. As a result, the abnormal sugar chain structure is based on the abnormality of glycosyltransferase. Glycosyltransferase catalyzes the chemical reaction of transferring monosaccharide part of active donor to saccharide and protein. β3GnT8 catalyzes the connection of GlcNAc in UDP-GlcNAc to Gal of Galβ1, 4Glc(NAc), thus forming polylactosamine sugar chain. CD147 is a highly glycosylated transmembrane glycoprotein, belonging to the immunoglobulin superfamily (member, participating in cell-to-cell adhesion and cell-to-matrix adhesion). It is highly expressed on the surface of various tumor cells, and plays an important role in cell proliferation, apoptosis, differentiation and tumor cell metastasis[16]. It can induce peripheral fibroblasts and tumor cells to produce various matrix metalloproteinases[17]. Studies have found that the effect of β3GnT8 on the behavior of tumor cells can be achieved by regulating the expression level of CD147[18-19]. The results showed that ursolic acid could inhibit the proliferation, adhesion and invasion of A549 cells in a concentration-dependent manner. Further study on the expression of glycosyltransferase β3GnT8 under the action of low and high concentrations of ursolic acid showed that with the increase of ursolic acid concentration, the expression of β3GnT8 decreased, and the expressions of CD147 and MMP2 also decreased at mRNA and protein levels. Therefore, ursolic acid could inhibit the adhesion and invasion of A549 cells by affecting β3GnT8.

To sum up, ursolic acid inhibits proliferation, adhesion and invasion of A549 lung adenocarcinoma cells, which is related to glycosyltransferase β3GnT8 gene. The mechanism is related to the down-regulated expressions of MMP-2 and CD147, and its specific mechanism needs further study. Ursolic acid can inhibit A549 lung adenocarcinoma cells by inhibiting the expression of β3GnT8 gene, which provides a new idea for anti-cancer treatment.

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