

Original Article

ASS1 expression in liver cancer tissues and effects of arginine deprivation on invasion and migration of liver cancer cells

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Abstract: Objective: To explore the expression of ASS1 in the liver cancer tissues, the effects of arginine deprivation on invasion and migration of the liver cancer cell lines and the relevant mechanism. Methods: The expression of ASS1 in liver cancer and normal liver tissues was detected by immunohistochemical assay; the expression of ASS1 in the cell lines HepG2, Hep3b, SMMC-7721, HB611 and BEL-7404 was detected by Western blotting; cell growth was detected by CCK-8 after different liver cancer cells were incubated with ADI-PEG20; the effects of ADI-PEG20 on invasion ability of the HepG2 cells were detected by Transwell invasion assay; the effects of ADI-PEG20 on migration ability of the HepG2 cells were detected by wound scratch assay; the expression of the proteins E-Cadherin, Vimentin and Twist was detected by Western blotting. Results: The expression of ASS1 was significantly reduced in liver cancer tissues compared to that in the normal liver tissues. As the pathological stage of liver cancer increased, ASS1 expression decreased gradually; with decrease in differentiation, ASS1 expression also decreased gradually; in liver cancer tissues with local metastasis, ASS1 expression was reduced significantly; in the five liver cancer cells, the expression level of ASS1 was HB611>SMMC-7721>BEL-7404>Hep3b>HepG2, with the lowest level in the HepG2 cells; the effects of ADI-PEG20 in liver cancer cells were negatively correlated with the expression level of ASS1; after incubation with ADI-PEG20, both invasion and migration abilities of the HepG2 cells decreased; incubation with ADI-PEG20 up-regulated the expression of E-Cadherin as well as down-regulated that of Vimentin and Twist. Conclusion: The expression of ASS1 was reduced significantly in liver cancer tissues and was closely related to tumor stage and grade and focal metastasis, and it has also been found out that ADI-PEG20 inhibits invasion and migration of liver cancer cells by inducing EMT.

Keywords: ASS1, liver cancer, arginine deprivation, EMT, invasion, migration

Introduction

Liver cancer is one of the most commonly seen malignant tumors in China, with about 150,000 people dying of the cancer each year, accounted for more than 50% of the total number of liver cancer deaths worldwide, posing a serious threat to people's health and life [1]. Metastasis and recurrence are the primary reasons for death of the patients with liver cancer. A number of studies [2, 3] have suggested that most of the patients have experienced micrometastases before treatment. Therefore, exploring the molecular biological mechanism of metastasis is of great importance in research on liver cancer, providing a new idea for diagnosis and treatment of the disease.

Tumor metastasis is a complex multi-step process involving many regulating factors. Current-

ly, it is known that tumor cells have undergone changes in adhesion ability, enhancement of angiogenesis, and cytoskeletal and kinetic characteristics as well as activation of various signaling pathways are involved [1]. The basis of metastasis is enhancement in the metabolic capabilities of tumor cells [4]. Therefore, determining the cell metabolism related molecular markers during the cancer metastasis process is not only of vital significance to elucidation of the metastasis mechanism, but also of important clinical and scientific value to evaluation of the clinical therapeutic effect and screening of relevant drugs.

Arginine is a non-essential amino acid for the human body, which is not only the raw material for synthesis of protein and nucleotide, but also the amino acid necessary for growth and prolif-

eration of tumor cells. Normal cells can take in arginine from serum as well as utilize citrulline to synthesize arginine [5]. Argininosuccinate Synthase 1 (ASS1) is the key rate-limiting enzyme in synthesizing arginine with citrulline. As is revealed in studies [6, 7] that in some tumors such as melanoma and renal cell carcinoma, ASS1 have a very low expression level or is not expressed. Arginine necessary for growth and proliferation of the tumor cells cannot be synthesized by the cells themselves but that in the serum must be relied on, which is called arginine auxotrophy. Therefore, as for the tumors with low or no expression of ASS1, the effect of killing tumor cells can be achieved through serum arginine deprivation.

As is shown in studies [8], using polyethylene glycol to wrap arginine deiminase (ADI) can effectively decompose arginine in serum, leading to arginine deprivation in the body. It has been demonstrated that [9-11] ADI-PEG20 has significant effects of killing tumor cells in tumors such as retinoblastoma and cancer of the kidney, pancreas and prostate. At present, however, there is no study on the expression of ASS1 in liver cancer or on the effects of arginine deprivation on invasion and migration of the cells.

Materials and methods

Collection and treatment of clinical samples

Liver cancer tissues of 70 patients confirmed with surgical resection and pathological examination at Henan People's Hospital from January 2015 to January 2016 were collected; in addition, normal liver tissues were collected from 70 patients after hepatic lobectomy for benign hepatic lesions. The patients were aged 62.45 ± 4.79 years. None of the patients had chemotherapy or radiotherapy before surgery. According to the TNM staging system for liver cancer (AJCC, the 7th edition), there were 22 patients at stage I, 10 at stage II, 36 at stage III, and 2 at stage IV; there were 44 patients with poor differentiation, 18 with moderate differentiation, and 8 with well differentiation. The tumor tissues isolated were divided into two pieces and fixed with 4% paraformaldehyde after blood stain was removed.

Cell lines and main reagents

The human liver cancer cell lines HepG2, Hep3b, SMMC-7721, HB611 and BEL-7404

were purchased from ATCC. Cell culture conditions: HepG2 and Hep3b were cultured in RPMI 1640 containing 10% fetal calf serum at 37°C, 5% CO₂. SMMC-7721, HB611, and BEL-7404 were cultured in RPMI DMEM containing 10% fetal calf serum at 37°C, 5% CO₂. The fetal calf serum, RPMI DMEM and RPMI 1640 medium were purchased from Gibco. The primary antibodies of ASS 1, E-Cadherin, Vimentin and Twist were purchased from Abcam (ab170952, ab40772, ab92547 and ab50887). Transwell chambers were obtained from Millipore (US).

Immunohistochemistry

The tissues were embedded in paraffin and cut into sections of 4 µm in thickness. The immunohistochemical operations were performed according to the immunohistochemical S-P kit (Beijing Zhongshan Golden Bridge Biotech Co., Ltd.): dewaxing of the tissues and then hydrating. Antigen retrieval was performed in microwave with citrate buffer solution for 30 min, cooled down to room temperature, then washed 3 times with PBS, 3 min each time, incubated with 3% H₂O₂ at 37°C for 15 min, then washed 3 times with PBS, 3 min each time, then the primary antibody was placed overnight, washed 3 times with PBS, 3 min each time; the horseradish peroxidase-labeled donkey anti-rabbit IgG (1:200, Beijing BIOSS Bio-tech Co., Ltd.), washed 3 times with PBS, 3 min each time; the horseradish peroxidase labeled streptavidin avidin working solution (Beijing BIOSS Bio-tech Co., Ltd.) was placed in 37°C water bath for 20 min, then washed 3 times with PBS, 3 min each time, and then stained with diaminobenzidine (DAB). In the negative control group, the primary antibody was replaced by PBS. All sections were reviewed independently by two pathologists, and 22 representative high power fields (10×40 fold) were selected by each pathologist. The percentage of positive cells in each specimen was counted and the positive results were judged and scored according to the method described by De Falco M et al. [12]: 0 (positive cells less than 1%); 1 (between 1% and 20%), 2 (between 21% and 40%); 3 (between 41% and 60%); and 4 (more than 61%).

Cell proliferation detected with CCK-8

Changes in cell proliferation were detected with CCK-8 after different liver cancer cell lines were treated with ADI-PEG20 at different concentrations. The 96-well plates were inoculated with 1×10^4 cells per well. There were HepG2, Hep3b,

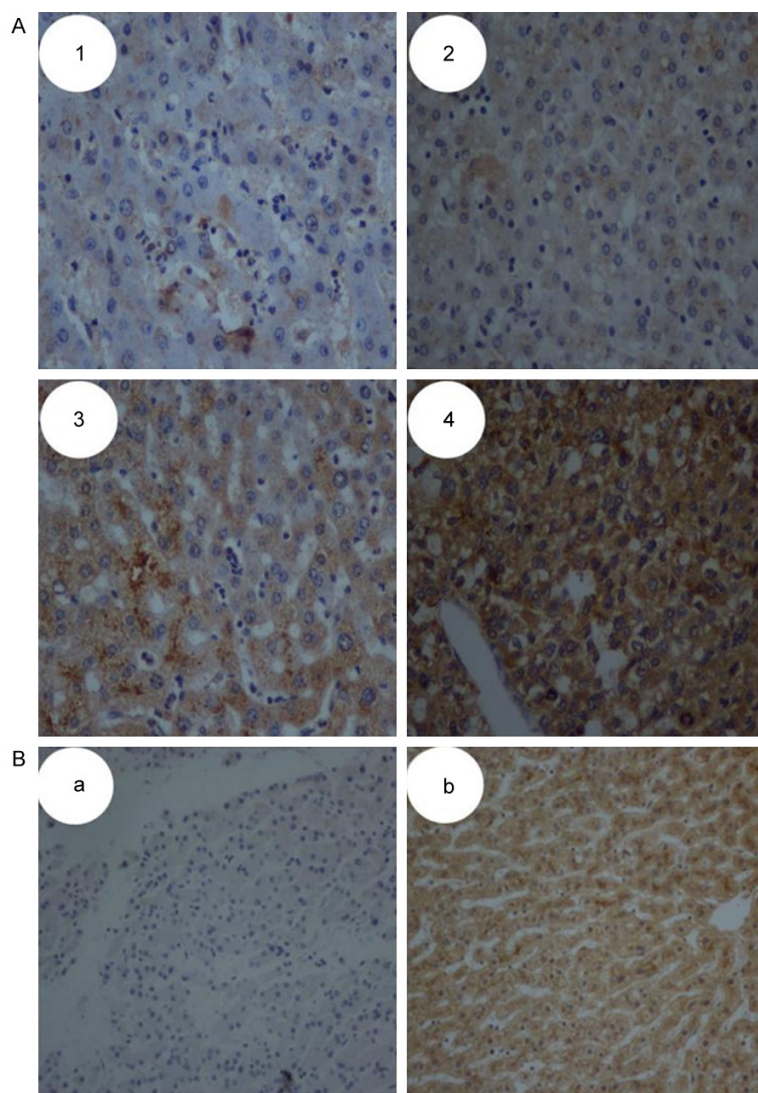


Figure 1. A. Representative images of 0 (positive cells less than 1%); 1 (between 1% and 20%); 2 (between 21% and 40%); 3 (between 41% and 60%); and 4 (more than 61%). B. Expression of ASS1 in liver cancer and normal liver tissues detected by immunohistochemical assay (10×). a. Expression of ASS1 in normal liver tissues; b. Expression of ASS1 in liver cancer tissues.

SMMC-7721, HB611 and BEL-7404 cell groups, 4 duplicate wells for each group. The cells were incubated in an incubator, growing to 50%~60% in cell density. 10 μ L of CCK-8 was added into each well to continue culture, and optical density (OD) was measured for each well at 490 nm 72 h later. The experiment was performed in triplicate.

Changes in cell proliferation were detected with CCK-8 after the HepG2 cells were treated with ADI-PEG20 at different concentrations. The 96-well plates were inoculated with 1×10^4 cells per well. There were ADI-PEG20 and NC groups, 4 duplicate wells for each group. The cells were

incubated in an incubator, growing to 50%~60% in cell density. 10 μ L of CCK-8 was added into each well to continue culture, and OD was measured for each well at 490 nm at 24, 48, 72, 96 and 120 h. The experiment was performed in triplicate.

Changes in cell invasion ability detected by transwell invasion assay after the HepG2 cells were treated with ADI-PEG20

All reagents and equipment were pre-cooled on ice. The Transwell chambers were placed in a 24-well plate. 50 μ L (0.2 μ g/ μ L) Matrigel gel was evenly applied to inner membrane of Transwell chamber, incubated for 15 min at 37°C to solidify the gel; when digested, centrifuged and counted, the cells were diluted with 2.5×10^4 /mL serum-free medium to prepare cell suspension; the cell suspension was added to the upper Transwell chamber at 200 μ L each well, and 500 μ L of 10% FBS and medium were added to the lower Transwell chamber, placed in a 37°C incubator for culture; fixed with formalin, stained by crystal violet for 15 min, and then the cells on the inner membrane were wiped with a cotton swab,

counted under a microscope, to count the cells that passed through the membrane under 4 high power fields ($\times 40$). The experiment was performed in triplicate.

Changes in cell invasion ability detected by wound scratch assay after the HepG2 cells were treated with ADI-PEG20

Wound scratch assay: The HepG2 cells were inoculated to a 6-well plate, and when cell confluence reached 90%, scratch from up to bottom using a 200 μ L sterile pipette tip, observe under a microscope, to measure the initial distance of scratch (0 time); at 24 h, 48 h and 72

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Table 1. Comparison of the expression of ASS1 between liver cancer and normal liver tissues

Tissue samples	Number	ASS1 protein positive	
		n	%
Liver cancer	70	14	20.0%*
Normal liver	70	53	75.7%

Compared with normal liver tissues, *P<0.001.

Table 2. Relationship between ASS1 expression and the clinicopathological characteristics of liver cancer

Clinicopathological data	Number	ASS1 positive	P value
Sex			0.703
Male	47	10 (21.27%)	
Female	23	4 (17.39%)	
Age (years)			0.337
≤60	38	6 (15.79%)	
>60	32	8 (25.00%)	
HBsAg			0.505
Positive	51	9 (17.64%)	
Negative	19	5 (26.31%)	
Differentiation			0.007
Well	8	5 (62.50%)	
Moderate	18	4 (22.22%)	
Poor	44	5 (11.36%)	
Pathological stage			0.037
I	22	8 (36.36%)	
II	10	3 (30.00%)	
III	36	3 (8.33%)	
IV	2	0 (0%)	
Local metastasis			0.041
No	38	11 (28.95%)	
Yes	32	3 (9.38%)	

h, the distances of scratch were measured respectively and photographed, to calculate the cell migration rate. The experiment was performed in triplicate.

Expression of the proteins ASS1, E-Cadherin, Vimentin and Twist detected by western blotting

The proteins were extracted from different liver cancer cell lines, and the protein concentrations were determined by BCA method, and then loading buffer was added for protein denaturation. 8% and 10% SDS-PAGE was prepared, and 20 µg protein sample was added into each

well, then transferred to a PVDF membrane using the electric wet transfer method, sealed 2 h with 5% skim milk, and the primary antibody (ASS1) was diluted by 1:1000 TBST, overnight at 4°C; then 1:5000 dilution of goat anti-rabbit secondary antibody was added, incubated at room temperature for 2 h; and ECL was performed. The experiment was performed in triplicate.

72 h after the HepG2 cells were treated with ADI-PEG20, the total proteins were extracted from the cells in the ADI-PEG20 and NC groups, and the protein concentrations were determined by BCA method, and then loading buffer was added for protein denaturation. 8% and 10% SDS-PAGE was prepared, and 20 µg protein sample was added into each well, then transferred to a PVDF membrane using the electric wet transfer method, sealed 2 h with 5% skim milk, and the primary antibodies (E-Cadherin, Vimentin and Twist) were diluted by 1:1000 TBST, overnight at 4°C; then 1:5000 dilution of goat anti-rabbit secondary antibody was added, incubated at room temperature for 2 h; and ECL was performed. The experiment was performed in triplicate.

Statistical analysis

The SPSS 19.0 software was used for statistical analysis, measurement data were expressed in ($\bar{X} \pm s$), t-test was employed for comparison of means between groups, and P<0.05 indicated statistically significant difference.

Results

Low expression of ASS1 in liver cancer tissues

The immunohistochemical results showed that ASS1 was localized in cytoplasm. In the liver cancer tissues of 70 patients, 14 were ASS1 positive; in the normal liver tissues of 70 patients, 53 were ASS1 positive (**Figure 1** and **Table 1**). It suggested that the expression of ASS1 was significantly reduced in the liver cancer tissues compared with that in the normal tissues, with statistically significant differences (P<0.001).

Relationship between ASS1 expression and the clinicopathological characteristics of liver cancer

The expression of ASS1 decreased as the pathological stage of liver cancer increased (**Table**

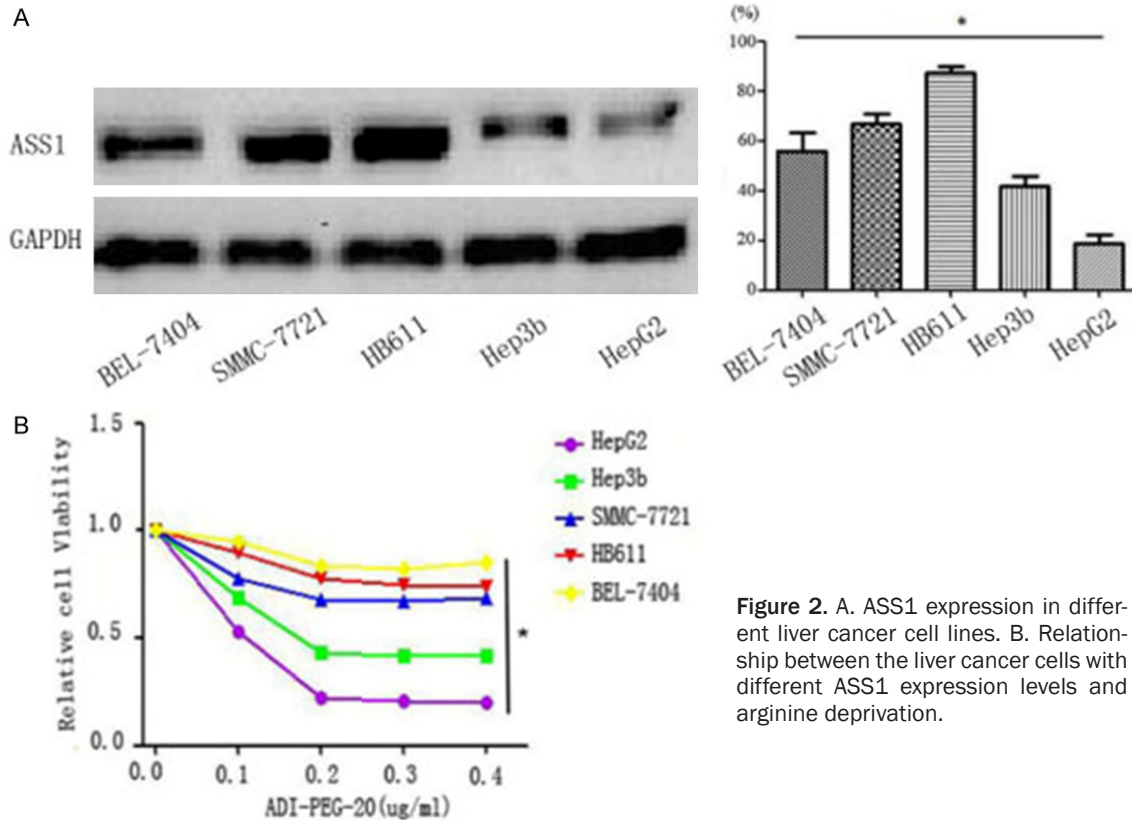


Figure 2. A. ASS1 expression in different liver cancer cell lines. B. Relationship between the liver cancer cells with different ASS1 expression levels and arginine deprivation.

2, $P=0.037$); with decrease in differentiation degree, the expression of ASS1 also decreased (Table 2, $P=0.07$); in liver cancer tissues with local metastasis, the expression of ASS1 decreased significantly (Table 2, $P=0.041$). The results indicated that the expression of ASS1 was related with pathological stage and grade of liver cancer and local metastasis.

Expression of ASS1 in different liver cancer cell lines

The Western blotting results showed that compared with the other liver cancer cell lines, expression of ASS1 in the HepG2 cells was the lowest ($54.2 \pm 2.14\%$ vs $65.3 \pm 2.42\%$ vs $83.19 \pm 4.19\%$ vs $39.91 \pm 1.45\%$ vs $18.79 \pm 1.21\%$), with statistically significant differences (Figure 2A).

Relationship between ASS1 expression and arginine deprivation

After treatment for 72 h of the liver cancer cell lines with various expression levels of ASS1 with ADI-PEG20 at different concentrations, the CCK-8 results showed that ADI-PEG20 had the strongest effect on the HepG2 cells with the lowest ASS1 expression level, and as the expression of ASS1 increased, the effect of

ADI-PEG20 decreased gradually. Arginine deprivation was the strongest when ADI-PEG20 was at a concentration of $0.2 \mu\text{g/ml}$ (Figure 2B).

ADI-PEG20 inhibits the invasion ability of the HepG2 cells

The Transwell results (Figure 3) showed that the number of cells passing the Matrigel gel was 44.92 ± 2.88 in the ADI-PEG20 group, obviously less than that in the NC group (181.54 ± 9.76), with statistically significant differences ($P < 0.001$). Results from the Transwell invasion assay indicated that ADI-PEG20 could inhibit invasion ability of the HepG2 cells.

ADI-PEG20 inhibits migration ability of the HepG2 cells

The width of scratches in any three parts of cells in each group was measured under a microscope at the time points of 0 h, 24 h, 48 h and 72 h. The migration rate was calculated according to the formula: $\text{Migration rate} = [D_{(t=24 \text{ h}, 48 \text{ h}, 72 \text{ h})} - D_{(t=0 \text{ h})}] / D_{(t=0 \text{ h})}$. Results of the wound scratch assay (Figure 4) suggested that compared to the NC group, migration rate in the ADI-PEG20 group was significantly reduced at 24 h, 48 h and 72 h [24 h (0.19 ± 0.03)%

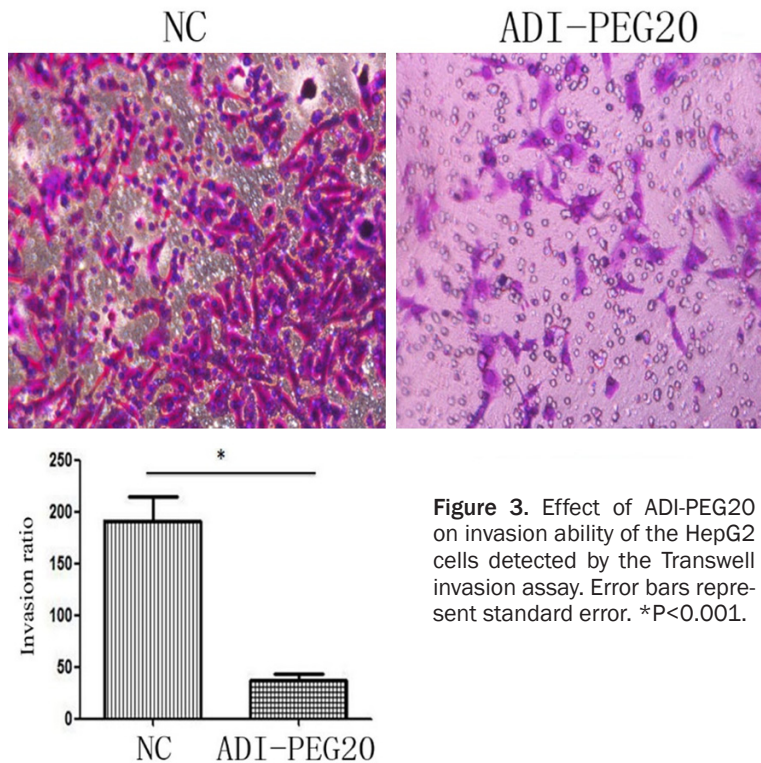


Figure 3. Effect of ADI-PEG20 on invasion ability of the HepG2 cells detected by the Transwell invasion assay. Error bars represent standard error. * $P < 0.001$.

vs $(0.34 \pm 0.04)\%$, $P < 0.05$; 48 h $(0.37 \pm 0.04)\%$ vs $(0.72 \pm 0.05)\%$, $P < 0.05$; 72 h $(0.44 \pm 0.04)\%$ vs $(0.91 \pm 0.06)\%$, $P < 0.001$], with statistically significant differences. It was revealed in the wound scratch assay that ADI-PEG20 could inhibit migration ability of the HepG2 cells.

ADI-PEG20 inhibits E-Cadherin, Vimentin and Twist expression

Many studies have demonstrated [13] that EMT is activated in invasion and migration of epithelial tumors, which is the critical molecular event enabling the epithelial tumor cells to acquire invasion ability and plays an important role in invasion and migration of malignant tumors. The E-Cadherin protein is the epithelial marker in the EMT process, while Vimentin and Twist are the mesenchymal markers in the process.

Results of Western blotting (**Figure 5**) showed that compared to the NC group, the expression levels of Vimentin and Twist in the ADI-PEG20 group decreased significantly [Vimentin $(0.28 \pm 0.01)\%$ vs $(0.77 \pm 0.06)\%$; Twist $(0.41 \pm 0.02)\%$ vs $(0.88 \pm 0.06)\%$, $P < 0.001$]; compared to the NC group, the expression level of E-Cadherin in the ADI-PEG20 group increased sig-

nificantly $[(0.86 \pm 0.06)\%$ vs $(0.41 \pm 0.02)\%$, $P < 0.001$]. It suggested that treatment of the HepG2 cells with ADI-PEG20 could up-regulate the expression of E-Cadherin, as well as down-regulate that of Vimentin and Twist. It indicated that ADI-PEG20 could promote EMT of liver cancer cells.

Discussion

ASS1 is a rate-limiting enzyme in synthesis of arginine, and disorder of ASS1 leads to the changes in arginine content. Arginine plays an important role in growth, proliferation, invasion, migration and angiogenesis of tumors. It has been suggested in study [14] that arginine added into the feeds of tumor-bearing mice increases the growth speed of tumor; and the lack of arginine in the feeds inhibits tumor metastasis.

This study has found that the expression of ASS1 was significantly reduced in liver cancer tissues than that in the normal tissues. As the pathological stage increased, the expression of ASS1 decreased gradually; with decrease in the differentiation degree, it also decreased gradually; in the liver cancer tissues with local metastasis, the expression of ASS1 decreased significantly.

Using arginine deiminase to transform arginine into citrulline for arginine deprivation can specifically kill the tumor cells without influencing the normal cells. Through investigation on the expression of ASS1 in different liver cancer cell lines, it has been demonstrated that ASS1 has the lowest expression level in the HepG2 cells; in addition, after using ADI-PEG20 to act on the different cell lines, the effects of ADI-PEG20 were negatively correlated with the expression level of ASS1, suggesting that the expression level of ASS1 was closely related with the effects of arginine deprivation. Wu et al. [15], by using siRNA to knock out the expression of ASS1 in breast cancer cells MCF-7, has found that this could increase its sensitivity to ADI-PEG20, consistent with the results of this study. Through further use of ADI-PEG20 to act on the

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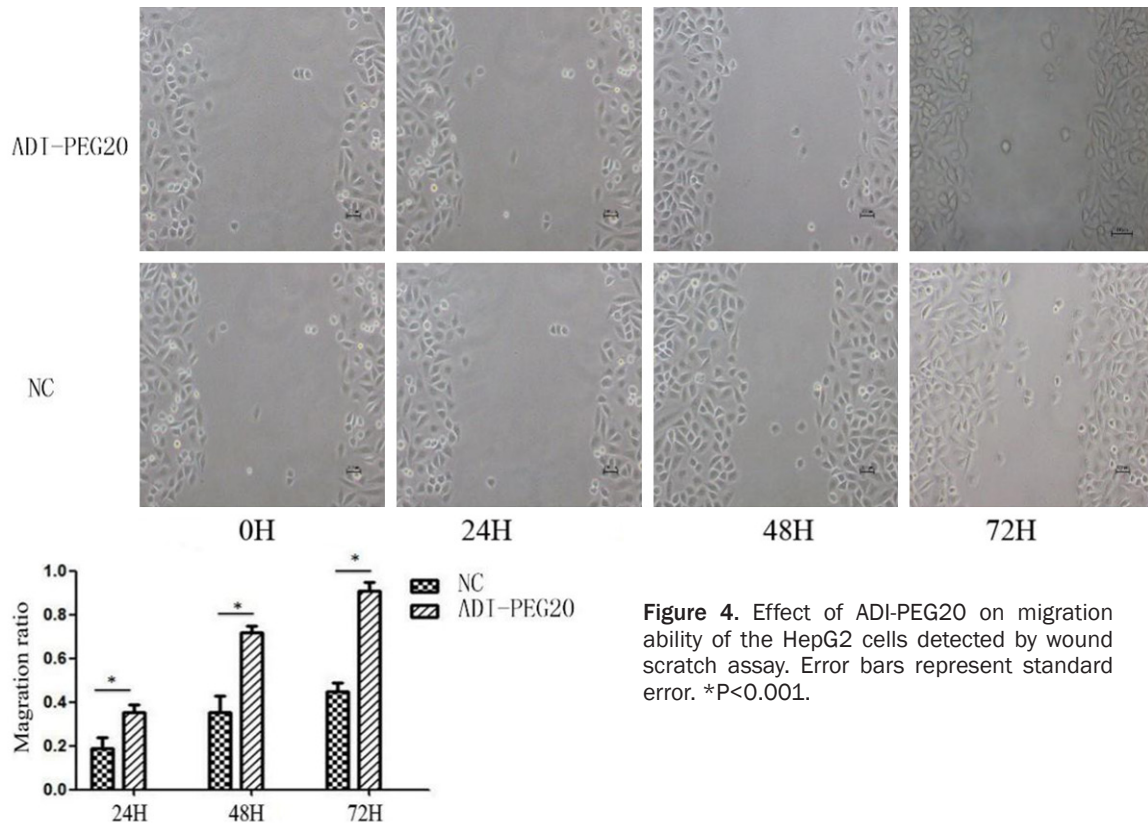


Figure 4. Effect of ADI-PEG20 on migration ability of the HepG2 cells detected by wound scratch assay. Error bars represent standard error. *P<0.001.

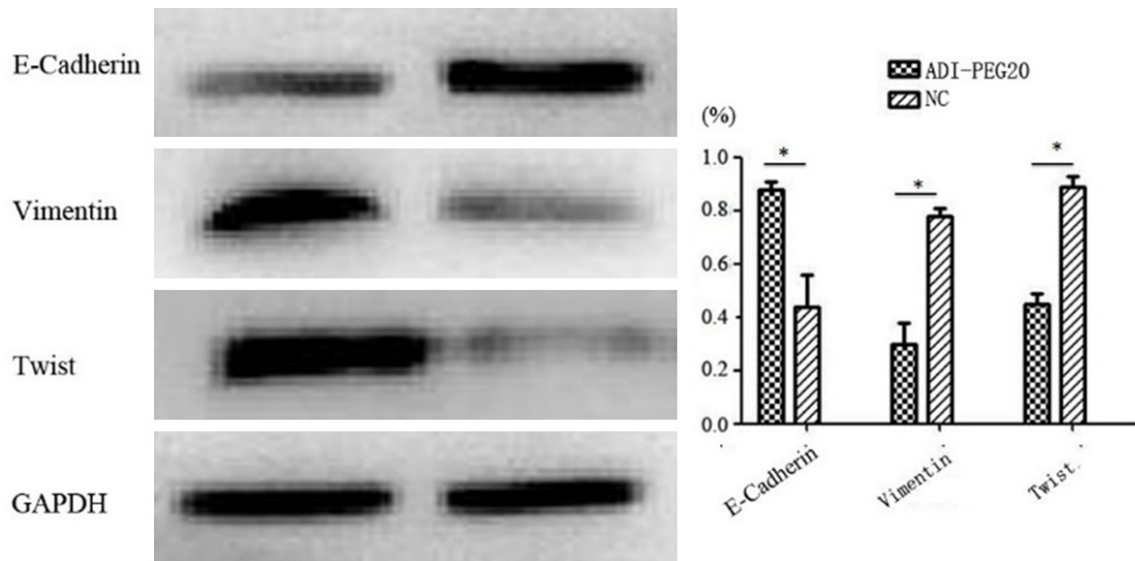


Figure 5. Expression of E-Cadherin, Vimentin and Twist detected by Western blotting. Error bars represent standard error. *P<0.001.

HepG2 cells, the invasion and migration abilities of the cells were investigated, and the results showed that after treatment with ADI-PEG20, the above abilities of the liver cancer cells were reduced.

EMT plays an important role in tumor infiltration and metastasis. The precondition for metastasis of tumor cells is that the adjacent tissues are infiltrated, the tumor adhesion is weakened to form highly invasive tumor and at the same

time, expression of E-Cadherin is down-regulated [16]. A number of studies have shown that expression of E-Cadherin is down-regulated in many malignant tumors, and the malignant tumors with low expression of E-Cadherin have stronger invasion and migration abilities. The study by Bonnoment et al. [17] has revealed that metastasis and invasion of tumor cells are increased significantly in the cells with EMT process. The study by Lee et al. [18] has revealed that over-expression of Twist in the liver cancer cells down-regulates expression of E-Cadherin, thereby inducing EMT. In this study, it has been observed that treatment of the HepG2 cells with ADI-PEG20 up-regulates the E-Cadherin expression and down-regulates the expression of Vimentin and Twist, suggesting that ADI-PEG20 can inhibit the invasion and migration abilities of liver cancer cells by inhibiting EMT in the cells.

This study suggests that ASS1 expression is significantly reduced in the liver cancer tissues and is closely related with tumor staging and grading and focal metastasis; meanwhile, it has been found out that ADI-PEG20 inhibits invasion and migration of liver cancer cells by inhibiting EMT in the cells. It indicates that ASS1 is likely to evolve into a personalized molecular target in the treatment of liver cancer.

Disclosure of conflict of interest

None.

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