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Original Article

Expression and biological roles of FAT10 in gallbladder cancer

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Abstract: Objective: We analyzed the abnormal expression, functional activation and molecular mechanism of human leukocyte antigen-F associated transcript 10 (FAT10) in gallbladder cancer. Method: Real-time PCR technique and Western Blot were applied to detect the expressions of FAT10 gene and protein in 80 cases of gallbladder cancer and the corresponding paracancerous tissues, respectively. Electroporation was performed to transfect the human gallbladder cancer cells GBC-SD with FAT10 siRNA and pcDNA3. 1-FAT10 recombinant expression vectors, respectively. Cell Counting Kit-8 was used to detect cell proliferative capacity; flow cytometry was performed to detect the changes in cell cycle distribution and cell apoptosis; cell invasiveness assay was performed to detect cell invasive capacity; RT-PCR and Western Blot were used to detect the changes of Hsp90/Akt signaling pathway. Result: Relative expressions of FAT10 gene and protein were significantly upregulated in the gallbladder cancer than in the paracancerous tissues ($P<0.05$). After the silencing of FAT10, the proliferative capacity of the gallbladder cancer cells decreased obviously, with downregulation of proteins related to Hsp90/Akt signaling pathway. In contrast, with FAT10 expression promoted by transfection, both the proliferative and invasive capacities of the cancer cells were enhanced considerably. No obvious changes were detected in cell cycle distribution, while the cell apoptosis rate decreased significantly and the expressions of proteins related to Hsp90/Akt signaling pathway were upregulated. Conclusion: FAT10 upregulated the expressions of proteins related to Hsp90/Akt signaling pathway. As a result, the proliferative and invasive capacities of the gallbladder cancer cells were enhanced and the cell apoptosis was reduced. FAT10 may be the potential target for the prevention and treatment of gallbladder cancer.

Keywords: Gallbladder cancer, FAT10, electrophoretic transfer, Hsp90/Akt signaling pathway, apoptosis, proliferation

Introduction

Gallbladder cancer ranks the first among all gastrointestinal tumors and even among general tumors in terms of incidence [1], and its incidence keeps rising every year [2]. Due to the lack of specific clinical symptoms, gallbladder cancer can be hardly differentiated even through surgical resection in some cases. Many cases are diagnosed in the middle to late stage and have missed the best timing for surgical treatment [3].

Though surgery is still the preferred treatment, the prognosis of gallbladder cancer is usually poor due to late diagnosis and high recurrence [4]. Non-surgical treatment, including chemotherapy and radiotherapy, is mainly auxiliary for middle-to-late stage cases. However, the treatment effect is uncertain, because many gallbladder cancers are resistant to radioactive rays and only respond mildly to chemotherapeutic agents [5, 6].

In recent years, genetic intervention provides an alternative treatment for cancers. Targeted therapy can directly regulate the expression of tumor-related molecules and modify the biological activities of tumor cells in a desired way. Gallbladder cancer is no exception [7, 9]. FAT10 is now considered the potential target for genetic interventions for cancers [10]. FAT10 plays a

role in cell proliferation, DNA repair, autophagy, signal transduction and embryonic development [11, 13]. Moreover, the abnormal expression of FAT10 has been observed in liver cancer, esophageal cancer, tongue cancer and colonic cancer [14-17].

The expression of FAT10 in gallbladder cancer and the mechanism are rarely studied. This paper aimed to analyze the expression, activation and molecular mechanism of FAT10 in gallbladder cancer. By silencing and activating the relevant gene, respectively, we evaluated the effect of FAT10 on the proliferative and invasive capacities of human gallbladder cancer cells. Moreover, the expressions of key molecules affecting the survival and apoptosis of the gallbladder cancer cells were characterized. The findings lay down the theoretical basis for the treatment of gallbladder cancer.

Materials and methods

Experiment design

Randomized controlled animal experiment.

Time and place of experiment

The experiment was carried out at Central Laboratory of Tianjin Medical University from February 2014 to September 2015.

Materials and reagents

Gallbladder cancer tissues and paracancerous tissues from 80 cases were provided by Central Laboratory of Tianjin Medical University. Trypsin was purchased from Sigma Inc. (USA). PBS buffer was provided by Hyclone (USA). Cell incubator was purchased from Heraeus Sepatech (Germany). FAT10 siRNA and pcDNA3. 1-FAT10 recombinant expression vectors were synthesized by Sangon Biotech (Shanghai) Co., Ltd. and donated by Prof. Caroline Lee from National University of Singaporem respectively. Trizol reagent was purchased from Invitrogen Corporation (USA). DMEM, fetal bovine serum (FBS) and PBS were purchased from Hyclone. BCA protein assay kit (enhanced), 5×SDS protein loading buffer and 20×TBS buffer were purchased from Nanjing Jiancheng Bioengineering Institute. FAT10, heat shock protein (Hsp) 90, Akt, and p-Akt antibody were purchased from Biomarker Technologies. β-actin antibody was purchased from ACON Biotech (Hangzhou) Co., Ltd. Normal saline was purchased from Tongji Hospital Affiliated to Shanghai Jiaotong University School of Medicine. The ultra-clean bench was purchased from Esco (Shanghai) Trading. Co., Ltd.

Method

Expressions of FAT10 gene and protein in gallbladder cancer tissue and paracancerous tissue

Gallbladder cancer tissues and the corresponding paracancerous tissues collected from 80 cases (male:female=24:56) were provided through the courtesy of Wuhan Union Hospital Affiliated to Huazhong University of Science and Technology. All specimens were fresh and collected from cases not receiving any anticancer therapies. RT-PCR was applied to detect the relative expression of FAT10 gene in the cancer tissues and the paracancerous tissues. Paired specimens from 8 cases were used to detect the relative expression of FAT10 protein by Western Blot.

FAT10 gene expression was detected using the method described by Wang [18] and Chen [19], with 3 replicates for each detection. The priers were designed using Primer Primer 5.0 software. For FAT10, upstream primer 5'-CAATGCTTCCTGCCTCTGTG-3', downstream primer 5'-TGCCTCTTTGCCTCATCACC-3'; for β-actin, upstream primer 5'-CCATCATGAAGTGTGACGTTG-3', downstream primer 5'-ACAGAGTACTTGCGCTCAGGA-3'. Total RNA extraction was performed. The relative expression of FAT10 gene was detected by RT-PCR. Using β-actin as internal reference, the relative expression of FAT10 mRNA was calculated by $2^{-\Delta\Delta Ct}$ method.

The expression of FAT10 protein was determined by Western Blot using the method by Gao et al [20, 21]. β-actin was used as the internal reference.

Transfection with FAT10 siRNA and pcDNA3. 1-FAT10

The gallbladder cancer cells were thawed, resuscitated and cultured using the method by Shi et al [22] and Ding et al [23]. The cells were cultured in high-glucose DMEM (dulbecco's modified eagle medium) containing 10% FBS and 100 U/mL penicillin-streptomycin at pH 7.4. Monolayer culture was performed at 37°C in a 5% CO_2 incubator, and the culture medium was replaced regularly. Cell passage was per-

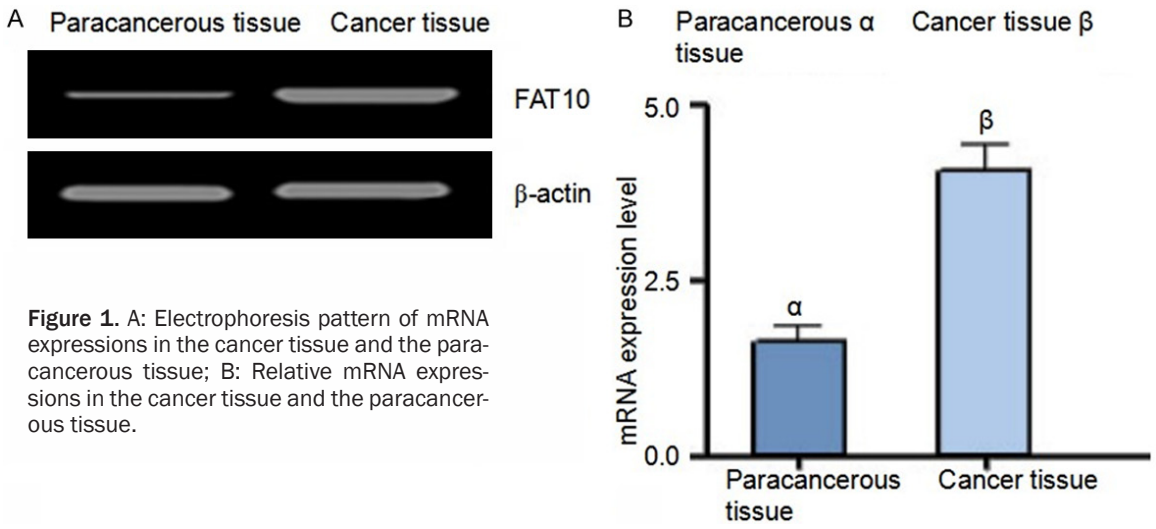

Figure 1. A: Electrophoresis pattern of mRNA expressions in the cancer tissue and the paracancerous tissue; B: Relative mRNA expressions in the cancer tissue and the paracancerous tissue.

Table 1. Relative expressions of FAT10 gene in the cancer tissue and the paracancerous tissue

	Relative expression of FAT10 gene	t	*P* value
Paracancerous tissue	1.631 ± 0.218		
Cancer tissue	4.076 ±0.342	4.012	<0.05

Note: Paired t-test for intergroup comparison, P<0.05.

formed when the cells grew to 80% confluence.

For transfection, the vigorously proliferating cells were divided into FAT10 siRNA transfection group, pcDNA3. 1-FAT10 transfection group and blank control group. All cells were digested with trypsin and resuspended in HeBS buffer (140 mmol/L NaCl, 5 mmol/L KCl, 0.75 mmol/L Na_2HPO_4, 6 mmol/L Glucose, 25 mmol/L Hepes). The cells were counted and adjusted to the density of 5×10^6/mL. Into the electroporation cuvette 200 μL of the cells were added. For each group, 4 μg FAT10 siRNA, normal saline and pcDNA3. 1-FAT10 were added, respectively. The cells were left to stand for 2 min at room temperature after electroporation. Next the cells were inoculated to three 6-well plates containing 2 ml of culture medium and three 96-well plates, respectively. All cells were cultured in an incubator.

Determination of proliferative and invasive capacities and apoptosis rate of cancer cells

The transfected cells were further cultivated for 24-48 h. Then the cells were detected for proliferative capacity by CCK-8 assay. The invasive capacity of the cancer cells was detected according to the literature [24, 25].

Propidium iodide (PI) staining was performed, followed by flow cytometry to determine the cell cycle distribution and apoptosis rate. First propidium iodide solution was prepared (containing 50 mg/L PI, 20 mg/L RNaseA, 1 g/L trisodium citrate, pH 7.4) and stored at 4°C in the dark. For each treatment, the cells were gently blown and resuspended in PBS buffer for several times to reach the density of 5×10^5/ml. The cells were fixed in 75% ethanol precooled at -20°C. Then the cells were left to stand at -20°C overnight. The fixed cells were centrifuged with supernatant discarded, and added with precooled PBS buffer. The cells were centrifuged again with supernatant discarded. RNaseA reagent was used to resuspend and digest the cells for 30 min. PI solution was added to stain the cells in the dark for 30 min at 4°C. After that, the flow cytometer was run to determine the cell cycle distribution based on DNA content in each phase of division as well as the apoptosis rate.

Changes of Hsp90/Akt signaling pathway after transfection

The transfected cells were further cultured for 24-48 h and then detected for the expressions of Hsp90, Akt and p-Akt genes and proteins by RT-PCR and Western Blot, respectively.

The mRNA expressions of the three proteins were determined according to the literature. The primers were designed by using Primer Primer 5.0 software and synthesized by Sangon

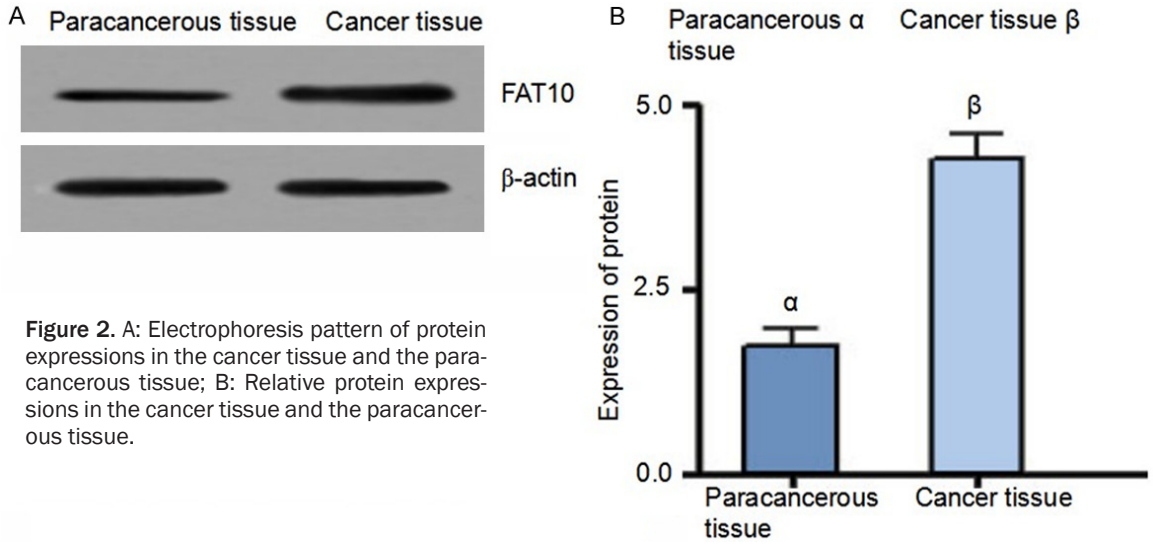

Figure 2. A: Electrophoresis pattern of protein expressions in the cancer tissue and the paracancerous tissue; B: Relative protein expressions in the cancer tissue and the paracancerous tissue.

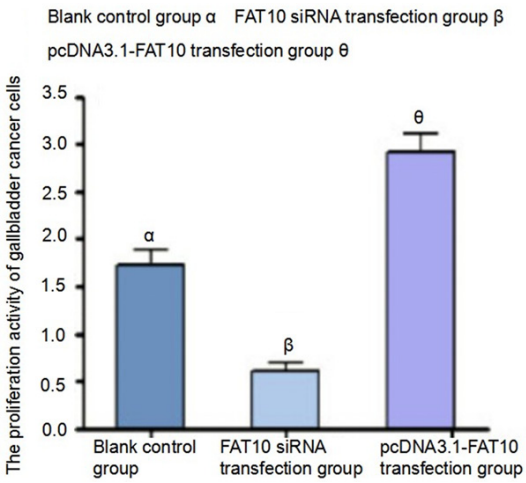

Figure 3. Proliferative capacity of the cancer cells.

Biotech (Shanghai) Co., Ltd. Total RNA was extracted and relative expressions of the genes were determined by RT-PCR. Using β-actin as internal reference, relative mRNA expressions of the three proteins were calculated by $2^{-\Delta\Delta Ct}$ method.

Protein expressions were determined by Western Blot according to the literature [26, 27]. First the transfected cells were digested by 0.025% trypsin to form the single-cell suspension. After protein quantification, the SDP-PAGE was performed. The proteins were transferred to NC membrane, which were sealed and incubated with primary AKT antibody (1:1000), primary p-Akt antibody (1:1000) and primary HSP90 antibody (1:1000), respectively. Relative expressions of the three proteins were analyzed after incubation.

Statistical analysis

All data were input into Excel 2010, with outliers removed. Statistical analysis was carried out using SPSS 16.0 software. One-way ANOVA was used for intergroup comparisons. The data were reported as mean ± standard deviation and $P<0.05$ indicated significant difference.

Results

Relative expressions of FAT10 gene and protein in gallbladder cancer tissue and paracancerous tissue

The relative expression of FAT10 gene was determined by RT-PCR. **Figure 1A** shows the electrophoresis pattern after RT-PCR. It can be seen that the target band corresponding to the cancer tissue was obviously wider than that of the paracancerous tissue. **Figure 1B** shows the mRNA expressions in the cancer tissue and the paracancerous tissue. **Table 1** shows the relative expressions of FAT10 gene in the cancer tissue and the paracancerous tissue. The relative expression of FAT10 was significantly higher in the cancer tissue than in the paracancerous tissue ($P<0.05$).

Eight paired specimens were used to determine FAT10 protein expression by Western Blot. As shown in **Figure 2A**, **2B**, the relative expression of FAT10 protein in the cancer tissue was obviously upregulated compared with the paracancerous tissue. Thus both FAT10 gene and protein were abnormally expressed in the gallbladder cancer tissue compared with the paracancerous tissue.

Table 2. Comparison of apoptosis rate between the groups

	Blank control group	FAT10 siRNA transfection group	pcDNA3. 1-FAT10 transfection group	*F* value	*P* value
Apoptosis rate	6.5 ± 1.3[a]	4.8 ± 0.7[b]	14.2 ± 1.6[a,b]	72.042	0.05

Note: P<0.05[a,b].

Table 3. Comparison of cell cycle distribution between the groups

Group	Cell cycle		
	G0/G1	G2/M	S
Blank control group	41.3 ± 4.8[a]	13.1 ± 1.8[a]	38.3 ± 1.8[a]
FAT10si RNA transfection group	51.1 ± 5.3[b]	12.3 ± 1.7[b]	28.3 ± 2.1[b]
pcDNA3. 1-FAT10 transfection group	73.2 ± 5.48[a,b]	6.41 ± 1.3[a,b]	18.1 ± 1.6[a,b]
F value	65.443	21.007	96.18
P value	0.05	0.05	0.05

Note: P<0.05[a,b].

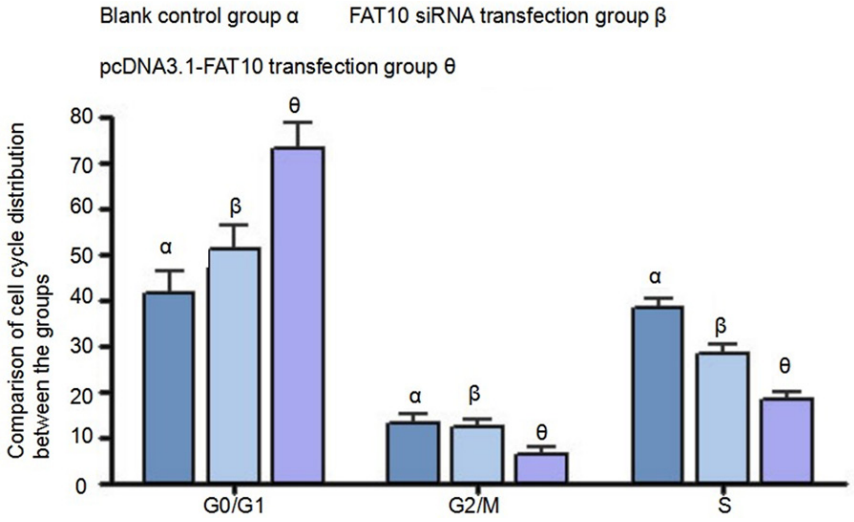

Figure 4. Comparison of cell cycle distribution between the groups.

Table 4. Changes in invasive capacity of GBC-SD cells

Group	Number of membrane penetrating cells
Blank control group	36.56 ± 2.11[a]
FAT10 siRNA transfection group	14.56 ± 2.06[b]
pcDNA3. 1-FAT10 transfection group	48.77 ± 2.35[a,b]

Note: P<0.05[a,b].

Changes in proliferative capacity, cell cycle distribution and apoptosis of GBC-SD cells after transfection

By transfection with siRNA and pcDNA3. 1-FAT10, the FAT10 expression was silen-ced and promoted, respectively. Then the proliferative capacity of the cancer cells was determined by CCK-8 assay. As sh-own in **Figure 3**, compared with the blank control group, siRNA transfection reduced the proliferative capacity of the cancer cells; in contrast, pcDNA3. 1-FAT10 transfection promoted the proliferative capacity of the cancer cells (P<0.05).

After transfection, the cell apoptosis rate and cell cycle distribution were de-termined using a flow cytometer. As shown in **Table 2**, siRNA silencing greatly increased the apoptosis rate of GBC-SD cells, whereas pcDNA3. 1-FAT10 transfection significantly reduced cell apoptosis. However, the distribution of cells arrested in G0/G1, G2/M and S phase did not change significantly between the three groups after transfection (**Table 3**; **Figure 4**).

Determination of invasive capacity of cancer cells

Table 4 shows the changes of invasive capacity of GBC-SD cells after transfection. The number of membrane penetrating cells in the blank control group, siRNA transfection group and pcDNA3. 1-FAT10 transfection group was 36.56 ± 2.11, 14.56 ± 2.06 and 48.77 ± 2.35, respectively. As shown in **Figure 5**, the silencing of FAT10 gene greatly reduced the invasive capacity of the cancer cells; in contrast, pcDNA3. 1-FAT10 transfection obviously promoted the invasive capacity of the cancer

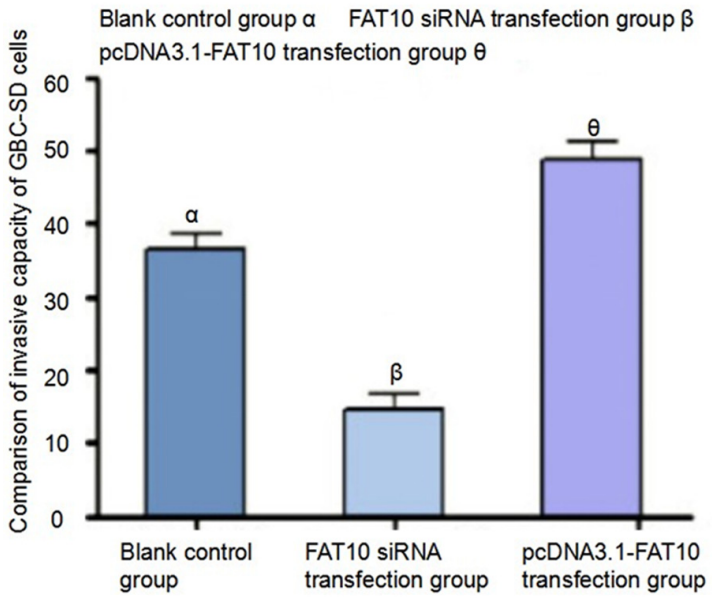

Figure 5. Comparison of invasive capacity of GBC-SD cells.

Table 5. mRNA expressions of HSP90, Akt and p-Akt

	FAT10 siRNA transfection group	pcDNA3. 1-FAT10 transfection group	Blank control group
HSP90	153.2 ± 1.38[b]	199.6 ± 2.11[a,b]	177.3 ± 2.11[a]
Akt	0.234 ± 0.157[b]	0.698 ±0.182[a,b]	0.634 ± 0.178[a]
p-Akt	0.256 ± 0.158[b]	0.842 ± 0.175[a,b]	0.745 ± 0.170[a]

Note: $P<0.05^{a,b}$.

cells. Compared with the blank control group, the number of membrane penetrating cells in the siRNA tranfection group decreased significantly, but increased significantly in the pcDNA3. 1-FAT10 transfection group. Thus, FAT10 played an im-portant role in promoting the invasive capacity of cancer cells.

Changes of Hsp90/Akt signaling pathway after transfection

We detected the mRNA and protein expressions of genes related to HSP90/Akt signaling pathway by RT-PCR and Western Blot, respectively. Three proteins were involved here, namely, HSP90, Akt, and p-Akt. According to **Table 5** and **Figure 6A**, **6B**, after the silencing of FAT10 gene by siRNA transfection, the mRNA expressions of the three proteins in the cancer tissue were significantly lower than those in the blank control group. In contrast, cDNA3. 1-FAT10 transfection greatly increased the mRNA expressions. The results of Western Blot detection are shown in Western Blot (**Table 6**; **Figure 7A**, **7B**). This indicated that FAT10 may play an important role in HSP90/Akt signaling pathway and participate in cancer cell survival and apoptosis.

Discussion

Ubiquitin plays an important role in the regulation of cell cycle, cell apoptosis and gene transcription [28]. As a member of the ubiquitin-like protein family, FAT10 is a regulator of cell cycle and involved in the immune process. At present, FAT10 is extensively studied as a potential target in anti-cancer therapy. FAT10 protein is expressed in gastrointestinal cancer, liver cancer, gynecologic cancer and pancreatic ductal cancer. It is generally held that the mechanism is related to mitotic chromosomal instability induced by binding to Mad2 [29, 30]. Moreover, downregulation of tumor suppressor gene p53 induced by high expression of FAT10 is another important reason of tumor occurrence and development [31]. We also found that FAT10 was abnormally expressed on the mRNA and protein level in human gallbladder cancer.

siRNA transfection and pcDNA3. 1-FAT10 transfection silenced and promoted FAT10 expression, respectively, thus indicating the role of FAT10 in sustaining the proliferation of gallbladder cancer cells. Instead of proving the regulatory role of FAT10 in cell cycle distribution [32], we did not observe a significant impact of FAT10 on cell cycle distribution of gallbladder cancer cells. Hu et al [33] also showed that FAT10 did not have a visible impact on cell cycle distribution in esophageal cancer cells, which agreed with our study. As the apoptosis rate of the gallbladder cancer cells increased by siRNA transcription, we inferred that FAT10 may enhance the survival of cancer cells by reducing cell apoptosis rather than by interfering with mitosis. Moreover, the invasiveness of the cancer cells increased significantly under high expression of FAT10. It is possible that ab-normal expression of FAT10 may contriute to the

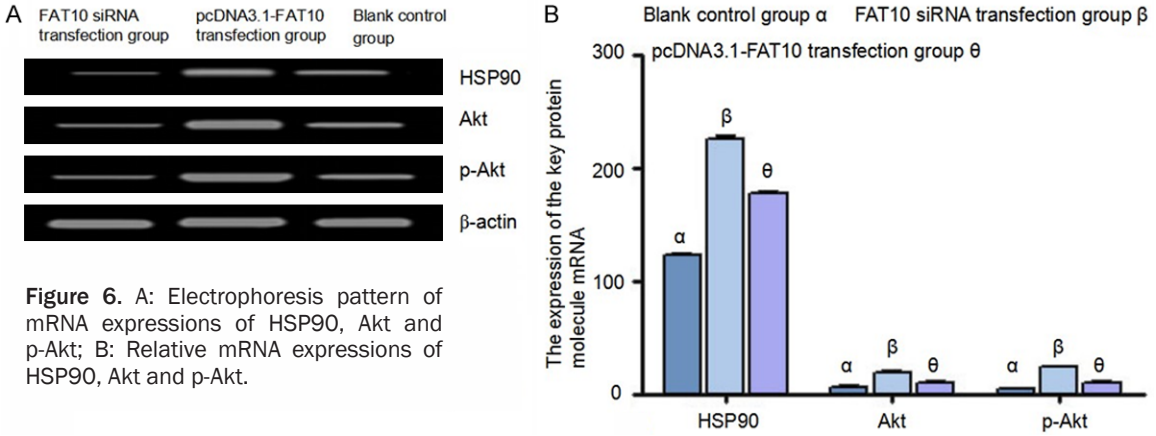

Figure 6. A: Electrophoresis pattern of mRNA expressions of HSP90, Akt and p-Akt; B: Relative mRNA expressions of HSP90, Akt and p-Akt.

Table 6. Protein expressions of HSP90, Akt and p-Akt

	FAT10 siRNA transfection group	pcDNA3. 1-FAT10 transfection group	Blank control group
HSP90	138.6 ± 1.27[b]	210.2 ± 1.35[a,b]	198.3 ± 1.30[a]
Akt	0.312 ± 1.09[b]	1.133 ± 1.27[a,b]	0.825 ± 1.21[a]
p-Akt	0.391 ± 1.08[b]	0.987 ± 1.21[a,b]	0.869 ± 1.06[a]

Note: $P<0.05^{a,b}$.

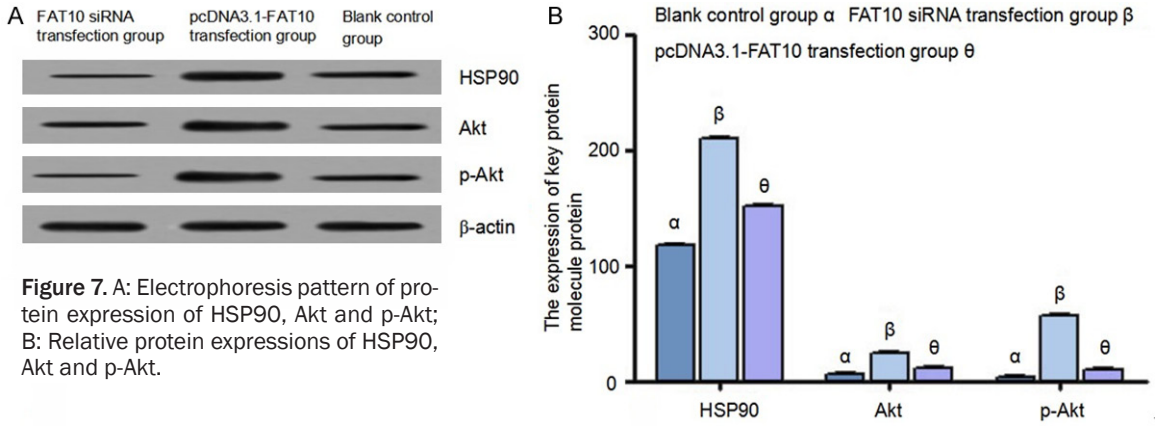

Figure 7. A: Electrophoresis pattern of protein expression of HSP90, Akt and p-Akt; B: Relative protein expressions of HSP90, Akt and p-Akt.

metastatic and invasive capacity of the cancer cells.

To prove the above hypothesis, we futher detected the expressions of proteins rel-ated to HSP90/Akt signaling pathway by silencing and promoting FAT10 gene, res-pectively. HSP90 co-ntains the chapeone-binding sites in the region between C-and N-terminals. By binding to client proteins, these sites en-sure the accurate fo-lding of the client protein into active conformation. So far over 100 client proteins have been identified for HSP90. Among them, Akt is the key regulator of cell apo-ptosis and survival. The downregulation of Akt and p-Akt will cause decreased ca-ncer cell apoptosis and invasiveness. The activated intracellular phosphatidylinositol-3 kinase (PI-3K), a key protein of the PI3K/Akt signaling pathway, will bind to Akt, thus inducing the membrane penetration of Akt and its phosphorylation. The activation of this signaling pathway will inhibit cell apoptosis and promote cell proliferation. It is found that PI3K/Akt signaling pathway is particular active in tumors, enhancing the invasive and metastatic capacities of tumor cells. Our results showed that high expression of FAT10 was conducive to the upregulation of HSP90-Akt/p-Akt on the mRNA and protein level, thus enhancing cell proliferation and invasiveness.

We characterized the mRNA and protein expressions of FAT10 in gallbladder cancer tissue and paracancerous tissue. By siRNA transfection and pcDNA3. 1-FAT10 transfection, we found that FAT10 promoted the proliferative and invasive capacities and reduced the apoptosis of gallbladder cancer cells by upregulating the proteins related to Hsp90/Akt signaling pathway.

Disclosure of conflict of interest

None.

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