Effects of Qingyi Huaji decoction on serum levels of interleukin-6, interleukin-8 and tumor necrosis factor-α in nude mice bearing pancreatic tumors

Hua-qiang Ouyang, Lu-ming Liu, Zhen Chen, Jian-min Luo, Er-xin Yu
Department of Integrated Traditional Chinese and Western Medicine, Cancer Hospital, Fudan University, Shanghai 200032, China

Objective: To investigate the effects of Qingyi Huaji (QYHJ) decoction, a compound traditional Chinese herbal medicine, on tumor inhibition rate and serum levels of interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) in nude mice with transplanted tumors of human pancreatic cancer.

Methods: The tumor-bearing mice model was established by subcutaneously inoculating with xenografts of pancreatic cancer into the right armpit of 40 BALB/c nude mice. After successful modeling, the mice were randomly divided into untreated group (Arabic gum), capecitabine group, low-dose QYHJ decoction group (36 g/kg) and high-dose QYHJ decoction group (72 g/kg), with 10 mice in each group. Citrate buffer solution (containing 5% Arabic gum), capecitabine suspension and QYHJ decoction were administered to four groups by gavage respectively. After 5-week treatment, the concentrations of serum IL-6, IL-8 and TNF-α were examined by enzyme-linked immunosorbent assay (ELISA) using blood sample from eye socket. Then the mice were euthanized by cervical dislocation. Tumor weight and the tumor inhibition rate were calculated.

Results: Tumor weight in the low-dose QYHJ decoction group decreased significantly as compared with the untreated group ($P<0.05$). Serum levels of IL-6 and TNF-α in low- and high-dose QYHJ groups were extremely significantly lower than those in the untreated group ($P<0.01$). Serum level of IL-8 in the low-dose QYHJ group was significantly lower than that in the untreated group ($P<0.05$). Correlation analysis showed that transplanted tumor weight of the mice was linearly positively correlated with serum levels of IL-6, IL-8 or TNF-α ($P<0.01$).

Conclusion: Conventional dose of QYHJ decoction is effective in suppressing pancreatic carcinoma in nude mice. The mechanism may be related to downstream regulation of serum cytokines such as IL-6, IL-8 and TNF-α.

Keywords: pancreatic adenocarcinoma; compound (TCD); interleukin-6; interleukin-8; tumor necrosis factor-α; mice, nude

清胰化积方对腺癌 CFPAC-1 移植瘤裸小鼠血清白细胞介素 6、白细胞介素 8 及肿瘤坏死因子 α 表达的影响

欧阳华强，刘鲁明，陈震，罗建民，于尔辛
复旦大学肿瘤医院中西医结合科，复旦大学上海医学院，上海 200032

目的：观察清胰化积（Qingyi Huaji，QYHJ）方对人腺癌 CFPAC-1 细胞系裸小鼠移植瘤的抑瘤作用及血清白细胞介素 6（interleukin-6，IL-6）、白细胞介素 8（interleukin-8，IL-8）和肿瘤坏死因子 α（tumor necrosis factor-α，TNF-α）的表达影响。

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Correspondence: Lu-ming Liu, MD, Professor; Tel: 021-64175590-3638; E-mail: lml1010@163.com
Pancreatic carcinoma is a common malignant tumor in digestive system with rapid progress and high incidence of metastasis. At the time of diagnosis, most patients with pancreatic carcinoma have already been at the advanced stage with a 5-year survival rate less than 5%\(^{[1]}\). A study in Cancer Hospital of Fudan University with 134 patients of advanced pancreatic cancer suggested an effective tumor inhibition role of Qingyi Huaji (QYHU) decoction, a compound traditional herbal medicine. The results showed that the 1-, 3- and 5-year survival rates were 25.0%, 14.1% and 8.4%, respectively, with a median survival time of 7.6 months. Sixteen patients survived for more than 3 years (5 survived for more than 5 years)\(^{[2, 3]}\). The survival rates were higher than the results in other similar studies with Western medicine. Although indicated with an effective tumor inhibition function, the mechanism of QYHU decoction has been further explored yet. It has been reported that serum cytokine levels and its immunomodulatory effects are closely related to the development of pancreatic cancer\(^{[4]}\). In this study, we investigated the effects of QYHU decoction on tumor inhibition and serum levels of interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) in nude mice with transplanted tumors of human pancreatic cancer.

1 Materials and methods

1.1 Experimental materials

1.1.1 Cell culture and animals Human pancreatic adenocarcinoma cell line CFPAC-1 was purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. According to the protocol of American Type Culture Collection (ATCC)\(^{[5]}\), the cell line was cultured in Iscoe’s modified Dulbecco’s medium (IMDM) supplemented with 10% fetal bovine serum, sodium pyruvate and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) in a humidified atmosphere containing 5% CO\(_2\) at 37 °C. Female 5-week-old BALB/c-nu/nu mice, SPF grade (Certification No. 122), weighing from 18 to 22 g, were purchased from Shanghai Experimental Animal Center of the Chinese Academy of Sciences.

1.1.2 Therapeutic medication QYHU decoction, composed of Baihuasheshcao (Herba Hedysotidis) 30 g, Banzhilian (Herba Scutellariae Barbatae) 30 g, Mou (Rhizoma Amorphophalli Rivieri) 30 g, Jiaogulan (Herba seu Radix Gynostemmatidis) 30 g and Baiduoukou (Fructus Amomi Rotundus) 6 g, was provided by Jiangyin Tianjiang Pharmaceutical Co., Ltd. (Jiangsu, China). According to the body surface area conversion table between humans and mice, herb medication were prepared in QYHU decoction groups at a dose of 36 g/kg or 72 g/kg, which including the crude drug of 1.8 g/mL and 3.6 g/mL, respectively, and oral administration was 0.4 mL/d per 20 g body weight for nude mice. Capecitabine (F. HoVman-La Roche, Shanghai, 0.5 g/tablet, certification No. H20073024) was crushed and dissolved in 100 mL citrate buffer (pH 6.0, 40 mmol/L) containing 5% (w/v) Arabic gum\(^{[6]}\).

1.1.3 Main reagents and instruments Sunrise F039300A microplate reader was provided by TECAN, Switzerland; enzyme-linked immuno-sorbent assay (ELISA) kits of IL-6, IL-8 and TNF-α were purchased from Shanghai Senxiong BioTechnology Co., Ltd., China; IMDM was purchased from Gibco Company, USA, (lot No. 12200-036); fetal bovine serum was provided by PAA Company, Austria (lot No. A15108-1479).

1.2 Methods

1.2.1 Preparation of mice model CFPAC-1 cells in logarithmic growth phase were prepared as single cell suspension at a concentration of \(1 \times 10^6\) cells/mL and were subcutaneously inoculated into the right flank of five nude mice at a dose of \(2 \times 10^6\) cells. Three weeks later, mice were sacrificed by cervical dislocation when the xenografts size reached 1 cm in diameter. Tumors were excised from mice, cut into small pieces and
transplanted subcutaneously into the right fore-limb armpit of another 40 nude mice.

1.2. Experimental grouping and medication
Seven days after transplantation, as the mean size of the tumors was about 0.4 cm in diameter, mice were randomly divided into untreated group (Arabic gum), capcitabine group, low-dose QYHJ decoction group (36 g/kg) and high-dose QYHJ decoction group (72 g/kg), with 10 mice in each group. For untreated group, citrate buffer solution (containing 5% Arabic gum) was administered by gavage, 0.4 mL per mouse, once a day for 5 weeks; for capcitabine group, capcitabine suspension 0.4 mL per mouse by gavage, once a day, 5 days a week, 3 weeks continuously; for low-dose QYHJ decoction group, 1.8 g/mL QYHJ 0.4 mL per mouse (36 g/kg), once a day for 5 weeks; for high-dose QYHJ decoction group, 3.6 g/mL QYHJ 0.4 mL per mouse (72 g/kg), once a day for 5 weeks.

1.3. Experimental detection
1.3.1. Observation of general condition Fur color, motility, nutritional status, body weight, mental condition and tumor size of the nude mice were observed and recorded daily.

1.3.2. Tumor volume changes
The maximal and minimal diameters of the tumor were measured by a vernier caliper once a week. Tumor volume and the inhibition rate were calculated according to the formula: \( V = \frac{1}{2} \times \text{maximal diameter} \times \text{minimal diameter} \) and inhibition rate of tumor volume = \( (1 - \text{average tumor volume of treatment group/average tumor volume of control group}) \times 100\% \). The growth curve was drawn according to the tumor volume measured daily.

1.3.3. Tumor inhibition rate
Mice were sacrificed 1 day after the last time of treatment. Tumor weight was estimated and the tumor inhibition rate was calculated. Tumor growth inhibition rate = \( (1 - \text{average tumor weight of treatment group/average tumor weight of control group}) \times 100\% \).

1.3.4. Detection of serum levels of IL-6, IL-8 and TNF-α
Concentrations of serum IL-6, IL-8 and TNF-α were examined by ELISA, using blood sample from eye socket which was stored at room temperature for 30 min, centrifuged (1000 × g) for 15 min, and then cryopreserved at -80 °C.

1.4. Statistical analysis
Statistical analysis was carried out with STATA software (version 10.0, College Station, Texas, USA). The measurement data were presented as \( \bar{x} \pm s \). One way analysis of variance was used to compare the differences between groups (symmetry transformation was used for non-normal distribution or variance non-homogeneity of the data), and the pairwise comparison was performed with Sidak method. Linear regression analysis was used to evaluate the correlation between cytokine level and tumor weights. Scatterplot graphs were created, and regression equations were built up. For all tests, \( P \) values less than 0.05 were considered statistically significant.

2. Results
2.1. General condition of the nude mice
During the first four weeks after successful modeling, nude mice in each group had balanced appearances, agile movements, flexible responses and increased body weights. As the tumor volume increased, life status of the mice worsened gradually, especially for untreated group. Two mice in the untreated group appeared as dark fur, loss of weight and energy at the fifth week. No deaths were observed until the mice were sacrificed. The whole body weight changes of nude mice showed an increasing tendency, however without significant differences between groups at each time point (\( P > 0.05 \), Table 1).

### Table 1. Body weight of nude mice in four groups

<table>
<thead>
<tr>
<th>Group</th>
<th>( n )</th>
<th>Dose (g/kg)</th>
<th>Average body weight in each group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Week 1</td>
</tr>
<tr>
<td>Untreated</td>
<td>10</td>
<td>0.36</td>
<td>22.5 ± 1.3</td>
</tr>
<tr>
<td>Capcitabine</td>
<td>10</td>
<td>0.36</td>
<td>22.0 ± 0.8</td>
</tr>
<tr>
<td>Low-dose QYHJ</td>
<td>10</td>
<td>36</td>
<td>22.3 ± 1.2</td>
</tr>
<tr>
<td>High-dose QYHJ</td>
<td>10</td>
<td>72</td>
<td>22.1 ± 1.3</td>
</tr>
</tbody>
</table>

2.2. Effects of QYHJ decoction on volume of transplanted tumors
Tumor with size of grain rice, could be detected immediately after the tumor pieces were subcutaneously transplanted into the nude mice. Primary tumor mass size was measurable after one week, with 100% successful modeling. At the initial stage, transplanted tumors were characterized with slow growth, spherical or hemispherical appearance, and movable. The tumors became harder gradually, with different individual size. After treatment, the average volumes of tumor in the capcitabine group, and low-dose and high-dose QYHJ groups were less than that in the untreated group, and the inhibition rates of tumor volume were 38.24%, 16.18% and 13.24% in those three groups respectively. There was no statistical difference for tumor volume between groups (\( P > 0.05 \), Table 2 and Figure 1).
Table 2  Tumor volumes and growth-inhibition rates of nude mice in different groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (g/kg)</th>
<th>n</th>
<th>Tumor volume ($\bar{x} \pm s$, cm³)</th>
<th>Growth-inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td></td>
<td>10</td>
<td>0.68±0.24</td>
<td></td>
</tr>
<tr>
<td>Capecitabine</td>
<td>0.36</td>
<td>10</td>
<td>0.42±0.14</td>
<td>38.24</td>
</tr>
<tr>
<td>Low-dose QYHJ</td>
<td>36</td>
<td>10</td>
<td>0.57±0.27</td>
<td>16.18</td>
</tr>
<tr>
<td>High-dose QYHJ</td>
<td>72</td>
<td>10</td>
<td>0.59±0.15</td>
<td>13.24</td>
</tr>
</tbody>
</table>

Figure 1  Growth curves of transplanted tumor in each group

Data were represented as $\bar{x} \pm s$, n=10.

2.3  Effects of QYHJ decoction on tumor weights

Tumor weight decreased significantly in the capcitabine and low-dose QYHJ groups as compared with the untreated group ($P<0.05$), while there were no significant differences between the high-dose QYHJ group and the untreated group ($P>0.05$) and between the QYHJ groups and the capcitabine group ($P>0.05$, Table 3).

2.4  Effects of QYHJ decoction on serum levels of IL-6, IL-8 and TNF-α

Compared with the untreated group, serum levels of IL-6 and TNF-α in the high- and low-dose QYHJ groups decreased significantly ($P<0.01$), and so did the serum IL-8 levels in the capcitabine and low-dose QYHJ groups ($P<0.05$). Compared with the capcitabine group, IL-6 and TNF-α levels in the high- and low-dose QYHJ groups also decreased significantly ($P<0.01$), however, IL-8 level in the low-dose QYHJ group increased without significance ($P>0.05$, Table 4).

Table 3  Tumor weights and growth-inhibition rates of nude mice in different groups

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Dose (g/kg)</th>
<th>Tumor weight ($\bar{x} \pm s$, g)</th>
<th>Growth-inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>10</td>
<td></td>
<td>0.61±0.16</td>
<td></td>
</tr>
<tr>
<td>Capecitabine</td>
<td>10</td>
<td>0.36</td>
<td>0.31±0.14</td>
<td>49.18</td>
</tr>
<tr>
<td>Low-dose QYHJ</td>
<td>10</td>
<td>36</td>
<td>0.40±0.22*</td>
<td>34.43</td>
</tr>
<tr>
<td>High-dose QYHJ</td>
<td>10</td>
<td>72</td>
<td>0.41±0.14</td>
<td>32.79</td>
</tr>
</tbody>
</table>

* $P<0.05$, ** $P<0.01$, vs untreated group.

Table 4  Contents of serum IL-6, IL-8 and TNF-α of nude mice in different groups ($\bar{x} \pm s$, ng/L)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Dose (g/kg)</th>
<th>IL-6</th>
<th>IL-8</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>10</td>
<td></td>
<td>29.06±6.99</td>
<td>200.75±50.80</td>
<td>29.50±5.09</td>
</tr>
<tr>
<td>Capecitabine</td>
<td>10</td>
<td>0.36</td>
<td>28.41±10.31</td>
<td>152.68±63.23*</td>
<td>25.59±4.97</td>
</tr>
<tr>
<td>Low-dose QYHJ</td>
<td>10</td>
<td>36</td>
<td>15.50±4.19*</td>
<td>169.85±77.67*</td>
<td>19.94±3.55* **△△</td>
</tr>
<tr>
<td>High-dose QYHJ</td>
<td>10</td>
<td>72</td>
<td>19.36±8.15**</td>
<td>193.50±48.04</td>
<td>20.89±2.43**</td>
</tr>
</tbody>
</table>

* $P<0.05$, ** $P<0.01$, vs untreated group; △△ $P<0.01$, vs capcitabine group.

2.5  Correlation of tumor weights and serum levels of IL-6, IL-8 and TNF-α

As mentioned above, there were statistical differences in serum levels of IL-6, IL-8 and TNF-α between the QYHJ groups and the untreated group. The relationships between transplanted tumor weight and serum levels of cytokines were further analyzed. The result showed a positive linear correlation between tumor weight and serum level of IL-6 in all groups ($R^2=0.32$, $P<0.01$). The regression equation for serum level of IL-6 and tumor weight was $y=12.8+23.58x$ (Figure 2, $x$ was tumor weight; $y$ was estimate of corresponding cytokine level, similarly hereinafter). There was also a positive linear correlation between serum level of IL-8 and tumor weight ($R^2=0.76$, $P<0.01$),
and the regression equation was $\hat{y} = 91.51 + 201.08x$ (Figure 3). The regression equation for serum level of TNF-α and tumor weight was $\hat{y} = 15.93 + 17.91x$ ($R^2=0.43, P<0.01$, Figure 4).

![Figure 2 Scatterplots of serum level of IL-6 and tumor weight](image)

**Figure 2** Scatterplots of serum level of IL-6 and tumor weight

![Figure 3 Scatterplots of serum level of IL-8 and tumor weight](image)

**Figure 3** Scatterplots of serum level of IL-8 and tumor weight

![Figure 4 Scatterplots of serum level of TNF-α and tumor weight](image)

**Figure 4** Scatterplots of serum level of TNF-α and tumor weight

3 Discussion

QYHJ decoction is a Chinese herbal compound based on the pathological mechanism of “noxious dampness, toxic heat and accumulation of pathogenic dampness, heat and toxin” for pancreatic cancer in traditional Chinese medicine. The product is made from Chinese herbs by using Moyu as monarch drug, which can reduce phlegm and resolve masses, detoxify and relieve swellings, dissipate blood stasis and relieve dyspepsia. Both Biahuasheshecao and Banzhilian are used as ministerial drugs, with the function of heat-clearing and detoxifying, removing dampness and deswelling, and activating blood analgesia. Jiaogulan is used as an adjuvant drug of strengthening healthy qi and disintoxication, resolving phlegm and anticancer. Baidoukou is used as the messenger drug for dissipation dampness to normalize stomach and promoting qi circulation to alleviate middle energizer. Clinical studies showed that QYHJ decoction was effective in advanced pancreatic cancer patients by heat-clearing and detoxifying, removing dampness and deswelling, regulating qi and dissipation blood stasis. We previously found that QYHJ decoction could inhibit the proliferation of human pancreatic cancer cell line SW1990 in vivo, and tumor weight in QYHJ group was significantly lower than that in the negative control group ($P<0.05$). When combined with gemcitabine, the tumor inhibition rate reached 92.69% to 93.41%, and tumor weight was significantly lower in the combined treatment group when compared with the gemcitabine group ($P<0.05$).

The results suggested that inhibition rates of tumor weight in the low- and high-dose QYHJ groups were 34.4% and 32.8% respectively, with statistical significance for the low-dose group when compared with the untreated group ($P<0.05$). However, inhibition rates of tumor volume were 16.2% and 13.2% respectively, without statistical significance between each QYHJ group and the untreated group ($P>0.05$). Moreover, heterogeneous changes were noticed in the capcetabine group and the QYHJ group with central necrosis, but no obvious tumor necrosis was detected in the untreated group. Therefore, disproportionate relation of tumor volume and weight may be due to internal necrosis in some tumor tissues or the possibility of measurement errors in the body surface.

The results showed that serum levels of IL-6, IL-8 and TNF-α in the QYHJ groups were significantly lower than those in the untreated group ($P<0.01, P<0.05$). TNF-α is also called cachectin. It is a monokine mainly produced by mononuclear macrophages, which can not only selectively kill some tumor cells and induce their apoptosis, but also has an effect of multiple immunoregulation. TNF-α receptors are widely distributed throughout tumor cells and blood cells. Binding of TNF-α and its receptors from some tumor cell surface of membrane may lead to death of these cells. High serum TNF-α level is an adverse prognostic factor for survival and event-free survival in patients with untreated acute myeloid leukemia or high-risk myelodysplastic syndrome. Talar-Wojnarowska et al studied 41 patients
with pancreatic adenocarcinoma, 56 with chronic pancreatitis and 50 healthy volunteers. Peripheral venous blood samples were obtained from all patients for TNF-α serum concentration measurement. The results showed that plasma level of TNF-α was significantly higher in pancreatic cancer patients (32.7 pg/mL) than in chronic pancreatitis patients (3.2 pg/mL) and healthy people (<1.6 pg/mL, P < 0.01). A positive correlation between weight loss in pancreatic cancer patients and serum level of TNF-α (P = 0.02) was also observed in their study. Patients with higher serum TNF-α level were more likely to develop cachexia. In the present study, serum TNF-α content in the untreated group was the highest, followed by capecitabine group. The results suggested that mechanism of capecitabine in the treatment of pancreatic cancer might have no relation to TNF-α level, which was similar to the conclusion made by Saif et al. in a phase 1 clinical trial of capecitabine and concurrent radiotherapy for locally advanced pancreatic cancer.

TNF-α has the function of killing or inhibiting cancer cells, but in current study we observed that the higher the serum TNF-α level, the heavier the tumor weight, which indicated an acceleration of TNF-α secretion accompanying with the growth of tumor. As the tumor volume increased, serum TNF-α level showed an increasing trend accordingly; and the decreased TNF-α level revealed that tumor was under control to a certain extent. Serum TNF-α level and its relationship with tumor progression remains to be elucidated.

IL-6 is a cytokine with the function of multiple immunoregulation. Many tumor cells may produce and secrete IL-6. Some studies showed that -174G/C IL-6 gene polymorphism influenced circulating IL-6 levels. Increased IL-6 serum level may be positively correlated with tumor size and the presence of liver metastases in patients with pancreatic adenocarcinoma (P < 0.01), and negatively correlated with serum albumin level. Overexpression of IL-6 usually means poor status and prognosis of cancer patients. IL-8 is also called CXC chemokine ligand-8, which is a cytokine mainly secreted by macrophages and epithelial cells, etc. IL-8 may promote tumor growth directly or through its role of stimulating angiogenesis. Significance of serum IL-8 in pancreatic cancer is similar to IL-6. Overexpression of IL-8 is closely related to weight loss, and these patients are prone to cachexia rapidly. However, some researches showed that the correlation of IL-8 and survival of patients was not so notable when compared with IL-6.

In the present study, serum IL-6 level in untreated group was the highest. No significant difference was found between the capecitabine group and the untreated group, while the difference was statistically significant in the low- and high-dose QYHJ groups as compared with the untreated group (P < 0.01), especially for the low-dose QYHJ group. The results indicated that conventional dose of QYHJ decoction could down-regulate the expression of serum IL-6 in CFPAC-1 transplanted tumor-bearing mice, but the downward effect was not directly proportional to dosage of QYHJ decoction. Moreover, we have observed that serum level of IL-8 was linearly positively correlated with transplanted tumor weight of the mice (R²=0.76, P<0.01), and relationship between tumor weight and IL-8 level was more close than its relation with IL-6 or TNF-α.

In the process of tumor development, high expression levels of cytokines such as IL-6, IL-8 and TNF-α are usually signals of progression of disease or poor prognosis. They are closely related to the occurrence of cancer cachexia. In the present study, serum IL-6, IL-8 and TNF-α levels in transplanted tumor of human pancreatic cancer cell line CFPAC-1 in nude mice were linearly positively correlated with tumor weight. As tumor weights in QYHJ groups were significantly lower than that in the untreated group, cytokine levels were correspondingly lower. The results demonstrated that serum levels of IL-6, IL-8 and TNF-α were important indicators for evaluating the sensitivity of pancreatic cancer to treatment of QYHJ decoction.

In conclusion, conventional dose of QYHJ decoction (36 g/kg) has an obvious antitumor effect on the subcutaneously transplanted tumors of human pancreatic carcinoma in nude mice. The mechanism may be related to down-regulation of cytokines such as IL-6, IL-8 and TNF-α.

**REFERENCES**


