

5-Fluorouracil concentration in blood, liver and tumor tissues and apoptosis of tumor cells after preoperative oral 5'-deoxy-5-fluorouridine in patients with hepatocellular carcinoma

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Abstract

AIM: To study the levels of 5-fluorouracil (5-FU) in plasma, liver and tumor in patients with hepatocellular carcinoma after oral administration of 5'-deoxy-5-fluorouridine (5'-DFUR).

METHODS: Thirty-nine patients with hepatocellular carcinoma were treated with oral 5'-DFUR for more than 4 d before operation. The contents of 5-FU in plasma, liver and tumor were measured by high performance liquid chromatography (HPLC) and apoptosis of tumor cells was evaluated by *in-situ* TUNEL after resection of tumor.

RESULTS: The concentrations of 5-FU were 1.1 µg/mL, 5.6, 5.9, and 10.5 µg/g in plasma, the liver tissue, the center of tumor and the periphery of tumor, respectively. 5-FU concentration was significantly higher in the periphery of tumor than that in the liver tissue and the center of tumor ($10.5 \pm 1.6 \mu\text{g/g}$ vs $5.6 \pm 0.8 \mu\text{g/g}$, $t = 21.38$, $P < 0.05$; $10.5 \pm 1.6 \mu\text{g/g}$ vs $5.9 \pm 0.9 \mu\text{g/g}$, $t = 20.07$, $P < 0.05$). 5-FU level was significantly lower in plasma than that in the liver and the tumor ($1.1 \pm 0.3 \mu\text{g/mL}$ vs $5.6 \pm 0.8 \mu\text{g/g}$, $t = 19.63$, $P < 0.05$; $1.1 \pm 0.3 \mu\text{g/mL}$ vs $10.5 \pm 1.6 \mu\text{g/g}$, $t = 41.01$, $P < 0.05$). Apoptosis of tumor cells was significantly increased after oral 5'-DFUR compared to the control group without 5'-DFUR treatment.

CONCLUSION: There is a higher concentration of 5-FU distributed in the tumor compared with liver tissue and apoptosis of tumor cells is increased following oral 5'-DFUR compared with the control group. The results indicate that 5'-DFUR is hopeful as neo-adjuvant chemotherapy to prevent recurrence after resection of hepatocellular carcinoma.

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Key words: Hepatocellular carcinoma; 5'-Deoxy-5-fluorouridine; 5-Fluorouracil; Apoptosis

INTRODUCTION

Hepatocellular carcinoma (HCC) remains one of the most common neoplasms in the world^[1,2]. 5'-Deoxy-5-fluorouridine (5'-DFUR) is an oral fluoropyrimidine derivative and it is converted to 5-fluorouracil (5-FU) by Pyrimidine nucleoside phosphorylase (PyNPase) which is expressed with higher level in tumor tissues compared with normal tissues^[3,4]. Oral 5-FU derivatives have shown comparable antitumor activity and long-term oral administration of low-dose has been considered as postoperative adjuvant chemotherapy after curative resection of the cancer to reduce recurrence and improve survival rate^[5-9]. The selective antitumor activity of 5'-DFUR is correlated with PyNPase activity in tumor^[10]. Although the activity of 5-FU in tumor is well recognized, resistance to this agent is frequently observed and remains its major limitation. It was reported that doxifluridine still showed antitumor activity to tumor cells which were resistant to 5-FU^[11].

The purpose of the present study was to investigate the impact of preoperative oral 5'-DFUR on distribution of 5-FU in plasma, liver and tumor and apoptosis of tumor cells in patients with hepatocellular carcinoma.

MATERIALS AND METHODS

Chemicals and reagents

5'-DFUR capsule (Furtulon) was produced by Shanghai Roche Pharmaceuticals Co. (Shanghai, China), and standard 5-FU was provided by Sigma Chemicals Co. (USA). Methyl-tert butyl ether, acetonitrile, methanol (HPLC grade) and potassium hydrogen phosphate (AR grade) were purchased from Peking Chemical Plant (Beijing, China).

Chromatographic conditions

HPLC system consisted of LC-10AT HPLC pump and SPD-10A Detector (SHIMADZU Co. Japan). The analytical column was Kromasil C₁₈ (5 µm 200 mm×4.6 mm ID). The mobile phase consisted of acetonitrile, methanol, and 0.25 mol/L potassium hydrogen phosphate (1:4:5). The

flow rate was 1.0 mL/min and the wavelength was at 265 nm.

Collection of samples

Thirty-nine patients of 27 male and 12 female with hepatocellular carcinoma were included in this study. Their average age was 49.1 years and the diameters of tumors were from 5 to 7 cm. 5'-DFUR was administered orally and preoperatively (1 200 daily for more than 4 d before operation and 400 mg on the day of operation). The mean total dosage was 6.4 g. Blood sample and specimens of the normal liver tissue and the tumor tissue were collected 3-5 h after final administration. The liver and tumor tissues were cleaned by distilled water and blotted by filter paper. One gram tissue was weighed accurately and 4 mL distilled water was added to the container to crush the tissue. The blood samples and the crushed tissues were centrifuged, and 1 mL plasma or aqueous layer sample was then stored under -20 °C.

Extraction and determination of samples

Each 0.5 mL plasma or aqueous layer sample was added and mixed with 0.1 mL of potassium hydrogen phosphate (0.5 mol/L), followed by the addition of 5 mL methyl-tert butyl ether to each tube. The tube was then capped and shaken for 10 min on a shaker. The aqueous layer and the organic layer were separated by centrifugation at 3 000 r/min for 10 min. The aqueous layer was transferred to a clean test tube and evaporated to dryness under gentle stream of nitrogen at 50 °C. The residue was dissolved with 0.1 mL of mobile phase and vortexed. Then the sample was transferred into a micro-spin filter tube and was centrifuged at 3 000 r/min for 10 min. The filtrate was collected and the injection volume was 20 µL and the content of 5-FU was measured by high performance liquid chromatography (HPLC)^[12-14].

In vivo detection of apoptosis

TUNEL staining was used to detect DNA degradation *in situ* in the relatively late stage of apoptosis. Apoptotic cells were labeled by the TUNEL reaction using an *in situ* Cell Apoptosis Detection Kit. *In situ* Cell Apoptosis Detection Kits were purchased from Roche Diagnostics GmbH Co. in Germany. The detailed manipulation was conducted according to instructions for users. The procedure was performed following the instructions of the manufacturer and in reference of the previous observations^[15,16].

The positive cells were identified and analyzed based on morphological characteristics of apoptotic cells as previously described. Under the fluorescence microscopy, apoptotic cells manifested as brownish staining in the nuclei. Non-necrotic zone was selected in the tissue section and images were randomly selected. The positive cells were determined in at least five areas at ×400 magnification and divided into three categories as follows: (+) only sporadic positive cells were detected; (++) a cluster of apoptotic cells were observed; (+++) positive cells in a large scale or multi-cluster apoptotic cells were seen in representative tissue sections of each individual case. Twenty patients of HCC without oral 5'-DFUR treatment were included as the control group.

Statistical analysis

The data were expressed as mean±SD. Student's *t*-test was performed for statistical analysis. *P* value less than 0.05 was considered statistically significant.

RESULTS

Concentration of 5-FU in plasma, liver and tumor following oral administration of 5'-DFUR

The concentrations of 5-FU were 1.1 µg/mL, 5.6, 5.9, and 10.5 µg/g in plasma, the liver tissue, the center of tumor and the periphery of tumor, respectively. The 5-FU content was significantly higher in the periphery of tumor than that in the liver tissue and the center of tumor (*P*<0.05). Moreover, the 5-FU concentration was significantly lower in plasma than liver and tumor (*P*<0.05). However, the 5-FU level in the center of tumor was similar to that in the liver tissue (Table 1).

Table 1 5-FU concentrations in blood, liver and tumor after oral 5'-DFUR

Tissue	Level of 5-FU
Plasma (µg/mL)	1.1±0.3 ^a
Liver tissue (µg/g)	5.6±0.8
Central tissue of tumor (µg/g)	5.9±0.9
Periphery tissue of tumor (µg/g)	10.5±1.6 ^a

^a*P*<0.05 vs 5-FU concentration in liver tissue.

Apoptosis of the tumor cell demonstrated by TUNEL

Under the fluorescence microscope, apoptotic cells manifested as brownish staining in the nuclei. The degree of apoptosis was shown + in tissue sections of the control group in which sporadic positive cells were detected, and ++ in cases with oral 5'-DFUR in which clusters of apoptotic cells were seen (Figures 1A and B).

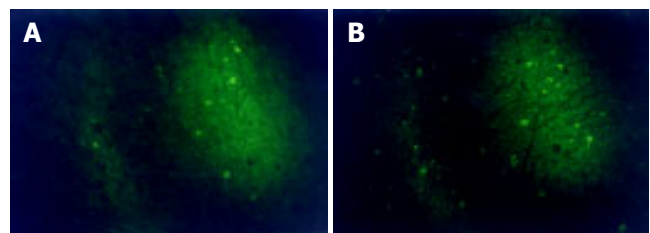


Figure 1 Apoptotic cancer cells detected by TUNEL method after oral administration of 5'-DFUR ×400. A: Sporadic apoptotic cells were detected; B: clusters of apoptotic cells were detected.

DISCUSSION

It is well known that hepatocellular carcinoma is one of the malignant tumors with poor chemosensitivity to anticancer agents^[17,18]. 5-FU is still the first choice for the chemotherapy of hepatocellular carcinoma^[19,20], because of its strong killing effects on the cancer cells. 5-FU can damage proliferating cells, reduce the tumor mass in size and prevent tumor

cells from spreading and metastasis. However, its usage is limited due to the rapid development of acquired resistance. The effects of 5-FU are not so satisfactory because 5-FU has a lower concentration in tumor tissue and relatively higher concentrations in blood after intravenous administration of 5-FU. Moreover, its side effects are serious and many patients are unable to tolerate.

5'-DFUR is a prodrug of 5-FU and it is converted to 5-FU by Pyrimidine nucleoside phosphorylase (PyNPase). PyNPase exists in all kinds of tumor tissues and its expression and activity in tumor tissue are higher than that in normal tissue^[3,4]. Nagata *et al.*^[21-24] reported that transfection of PyNPase gene into tumor cell can increase the sensitivity to 5'-DFUR, and thereby decreases the toxicity of the agent. In our study, it had been found that 5-FU level in tumor was 10 times higher than in plasma and 5-FU level was significantly higher in the periphery of tumor than in the liver tissue. The results suggested that more 5-FU was converted and accumulated within tumor tissue. This difference may be related to the higher PyNPase expression and activity in hepatocellular carcinoma. It had also been found that the 5-FU level was significantly higher in the periphery of tumor than the center of tumor. There was more 5-FU accumulated and converted in the periphery of tumor following oral 5'-DFUR administration. Oxygen, nutrition and growth factors were not equally distributed within the tumor tissue. Oxygen and nutrient of the central tumor tissue are supplied mainly by hepatic artery, and proliferation of the tumor in this region is slower and even partial necrosis appears. However, the periphery of tumor has effluent blood flow supplied by hepatic artery and portal vein, so the tumor cells can grow more rapidly. These results may indicate that large liver tumor is not sensitive to 5'-DFUR.

Oral 5'-DFUR is very convenient and its side effect is slight^[25]. The drug arrives in the liver firstly after being absorbed by the intestine. So most of 5'-DFUR can be converted to 5-FU in the liver or the liver tumor and accumulated in these tissues with higher concentration than that administered by iv approach. In this study, the 5-FU level in peripheral vein was only one-tenth of that in tumor tissue. 5-FU can be maintained at a certain level within the tumor tissue but not accumulated in the peripheral vein after regular oral administration of 5'-DFUR.

The expression and activity of PyNPase is higher in the tumor with vascular permeation and lymph node metastasis^[4,26,27]. Akao *et al.*^[28] reported that PyNPase activity was significantly higher in metastatic lymph node. Tazawa *et al.*^[29] found that 5'-DFUR could inhibit hepatic metastasis of tumor, which would be effective for the prophylactic treatment of metastatic disease. These indicate that PyNPase activity appears to be a new useful parameter for identifying both a poor prognosis and a highly malignant potential of tumor and 5'-DFUR is preferably sensitive in cancer patients with lymph node metastasis and vascular infiltration. 5'-DFUR is still effective to the tumor which is resistant to 5-FU therapy^[11]. Monden *et al.*^[30] found that vessel density and stage of tumor were correlated with expression of PyNPase which showed prognosis. 5'-DFUR not only is effective on primary tumor and metastatic lesion but also

suitable to postoperative prevention of tumor. Apoptosis of the tumor was enhanced after oral administration of 5'-DFUR. In this study, it was found that there were more apoptosis cells in the tumor after oral administration of 5'-DFUR.

In summary, our results showed that there was a higher concentration of 5-FU accumulated in the tumor tissue compared with liver tissue and apoptosis of the tumor cells was increased after oral administration of 5'-DFUR. 5'-DFUR is a hopeful agent for neo-adjuvant chemotherapy to prevent recurrence after resection of hepatocellular carcinoma.

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