Thymidine phosphorylase induced by IFN-alpha2b enhances 5-fluorouracil antitumor activity *in vitro* and *in vivo*

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Abstract

Objective. To explore the effect of thymidine phosphorylase expression induced by interferon- alpha on chemosensitivity of 5-fluorouracil(5-FU) in renal clear cell carcinoma. *Methods*. Renal clear cell carcinoma cell line 786-0 was treated by various concentration of interferon (IFN)-alpha2b *in vitro*, and semi-quantitative RT-PCR was used to determine TP mRNA expression. Western blot was used to determine TP protein expression. 50% inhibitory concentration of growth (IC_{50}) of 5-FU in different groups of treated 786-0 cell line was evaluated by MTT assay. The athymic mouse model of xenograft renal clear cell carcinoma were established, and effect of chemotherapy of IFN-alpha2b combined with 5-FU was examined on the growth of xenograft tumor. *Results*. IFN-alpha2b has promoted TP mRNA expression in a dose dependent manner and increased TP protein expression($P<0.01$). IC_{50} of 5-FU to 786-0 cell line was obviously decreased with IFN-alpha2b($P<0.01$). Effect of chemotherapy of 5-FU was obviously enhanced *in vivo* after IFN-alpha2b combined with 5-FU($P<0.05$). *Conclusion*. Up-regulating expression of TP induced by IFN-alpha2b has involved in enhanced cytotoxicity of chemotherapy. [Life Science Journal. 2007;4(1):17-20] (ISSN: 1097-8135).

Keywords: thymidine phosphorylase; interferon; renal clear cell carcinoma; 5-fluorouracil

1 Introduction

Most renal carcinoma patients are insensitive to chemotherapy. Several clinical studies proved certain cytokines combined with chemotherapy can greatly increase chemosensitivity to renal carcinoma cells[1]. At present 5-fluorouracil (5-FU) and interferon-alpha (IFN-alpha) have applied in clinical treatment. In some cases combination of 5-FU and IFN-alpha have synergized but the underlying mechanism is still unclear. Thymidine phosphorylase (TP) is a nucleoside metabolism enzyme. Compared with adjacent non-neoplastic tissues, higher TP expression is observed in a wide variety of solid tumors including renal cell carcinoma. It is the rate-limiting enzyme of 5-FU activation in tumor and associated with catabolism of 5-FU *in vivo*[2]. In present study, renal clear cell line 786-0 was treated by various concentrations of IFN-alpha2b to explore the mechanism of synergistic effect its through detecting TP expression and its sensitivity to 5-FU.

2 Materials and Methods

2.1 Materials

IFN-alpha2b (1.8×10^7 IU/1.5 ml) were provided by Schering-Plough (China) Ltd. 5-FU were purchased from Sunrise(Shanghai, China). 786-0 cells, human renal clear carcinoma cell line, were obtained from the cell Bank of Chinese Academy of Science (Shanghai, China) and the culture medium was RPMI1640 supplemented with fetal serum (10%) and antibiotics. Female BALB/c Mice 4-6 weeks old weighting approximate 20 g (Institute of laboratory Animal Science, Chinese Academy of Science, China) were housed in laminar flow cabinets under specific pathogen-free conditions.

2.2 Detection of TP mRNA by RT-PCR

786-0 cells were plated on six-well plates with 1×10^6 in each well and cultured overnight before treated with IFN-alpha2b. Cells were divided into five groups numbered 1 to 5 and treated with 0, 1000, 3000, 6000 and 12000 IU/ml of IFN-alpha2b respectively for 72 hours. Total RNA of each group was extracted using the Trizol according to the manufacturer's instructions (Invitrogen Corp, England). Complementary DNA (cDNA) was synthesized using AMV RT system (Sangon, China) with 2 μg of total RNA from each of group with the final volume of 20 μl. The expression of TP mRNA was semi-quantitatively evaluated by PCR amplification using primer: TP: forward 5′-CATCCAGAGCCCAGAGCAGA-3′, reverse 5′-CCGAACTTAACGTCCACCACC-3′. glyceraldehyde-3-phosphate dehydrogenase(GAPDH) was as an internal standard. GAPDH: forward: 5′-GCACCGTCAAGGCTGAGAA-3′, reverse:

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5′-AGGTCCACCACTGACACGTTG-3′. PCR reactions for two genes were carried out together by TaKaRa Taq™(Takara, China). Each cycle consisted of denaturation at 94 ℃ for 40 seconds, annealing at 54 ℃ for 45 seconds, and extension at 72 ℃ for 50 seconds. Negative-control PCR was conducted using an aliquot from control RT reaction (in the absence of reverse transcriptase) as a template, and showed no PCR product. PCR products were separated by electrophoresis on 1.5% agarose gels and stained with ethidium bromide. Band intensity was determined with SYN GENE Image picture analyzing software. Expression of TP mRNA was normalized to that of GAPDH.

2.3 Detection of TP protein expression by Western blot

Treated cells were harvested and lysed in lysis buffer. Protein concentrations were measured using the Bradford method. 50 μg protein was loaded per lane and separated by 10% SDS-PAGE gel and electroblotted onto nitrocellulose membranes. The membranes were blocked in 5% skimmed milk powder diluted in TBST. The membrane was then incubated with anti-TP antibody (Santa Cruz, American.) at 1 : 500 dilution overnight. Subsequently, membranes were incubated with a secondary horseradish peroxidase (HRP)-conjugated antibody in wash buffer (0.2% skimmed milk powder diluted in TBST) at 1: 5000 dilution for 2 hours. Finally immunolabeled proteins were visualized using DAB according to the manufacturer's instructions (Zsbio, China). As a loading control, the blot was incubated and reprobed with anti-β-actin antibody (Santa Cruz, American).

2.4 Drug sensitivity *in vitro*

Rapidly growing cells were seeded in 96-well plates with density of 5×10^4 cells/well. After 24 hours, cells were divided into 5-FU group, combination group and control group. In 5-FU group and combination group, a RPMI 1640 medium containing 5-FU from 1 to 10^5 μmol/L was added to appropriate wells. Simultaneously IFN-alpha2b was added into each well in combination group at 6000 IU/ml concentration. 8 wells that were added RPMI 1640 medium without any drugs were taken as control group. The plates were incubated at 37 ℃ in a 5% CO_2 incubator for 72 hours. 50 μl MTT (5 mg/L) were added into each well and after incubation for another 4 hours, supernatant medium were replaced by 150 μl DMSO. The plates shaken for 10 minutes before absorbance at 492 nm were measured with a microplate reader. The inhibition rate was calculated with the formula: % inhibition = [1 − (absorbance of experimental wells/average absorbance of control wells)] × 100%. Dose-response curves were plotted, and the 50% inhibitory concentration (IC_{50}) was determined graphically.

2.5 Antitumor effect *in vivo*

The antitumor effect of 5-FU alone or combined with IFN-alpha2b was investigated in 15 xenograft-bearing BALB/c mice. Each animal was subcutaneously transplanted with 2×10^6 786-0 cells suspended in 200 μl PBS. 15 days after tumor graft, animals in 5-FU group were received 5-FU (25 mg/kg, *q.o.d*, *i.p.*). Animals in combination group were received the same dosage of 5-FU and IFN-alpha2b (3×10^5 IU/mouse, daily, *i.m.*). Animals in control group were received saline (*i.p.*). Injections were performed for 4 weeks and all animal were executed and tumor weights were scaled with a digital balance.

2.6 Statistical analysis

The results were expressed as the mean ± SD. Differences between groups were analyzed using either one-way ANOVA or one-way ANOVA on ranks with the least significant difference(LSD) tests. P value of 0.05 was regarded as significant. All analysis were performed using SPSS10.0 software.

3 Results

3.1 Effect of IFN-alpha2b on the expression of TP mRNA

The results of RT-PCR were shown in Figure 1. Expression quantification of group 1 to 5 were 0.5133 ± 0.0152, 0.5667 ± 0.0115, 0.5733 ± 0.0231, 0.8233 ± 0.0404, 0.8366 ± 0.0451, respectively. There were significantly statistical differences between these groups ($F = 78.493$, $P < 0.01$). LSD analysis showed TP mRNA expression increased significantly when treated concentration of IFN-alpha2b is more than 6000 IU/ml. The expression of TP mRNA was significantly increased in a dose-dependent manner with the IFN-alpha2b concentration($r = 0.901$, $P < 0.05$).

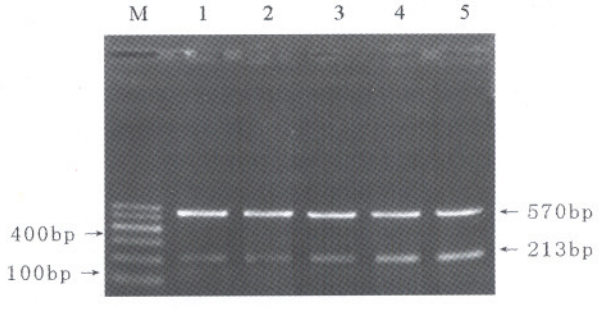

Figure 1. Results of IFN-alpha2b on the expression of TP mRNA Concentration of IFN-alpha2b (IU/ ml): Lane 1: 0; Lane 2: 1,000; Lane 3:3,000; Lane 4:6,000; Lane 5:12,000; Lane M: Marker

3.2 Effect of IFN-alpha2b on the expression of TP protein

Expression levels of TP protein were shown in Fig-

ure 2 by Western blot. A band at approximate 45 kDa was detected on the immunoblots, which corresponds to TP protein. Compared with control group, group 2 with 3,000 IU/ml IFN-alpha2b and group 3 with 6,000 UI/ml IFN-alpha2b were indicated stronger expression of TP protein. Quantification were 0.6167 ± 0.0611, 0.6347 ± 0.0719, 0.8735 ± 0.0640 in 1, 2 and 3 group respectively with software Totallab 2.0. Increase of TP protein expression in group 3 was significant compared with control group($F = 14.232, P < 0.01$).

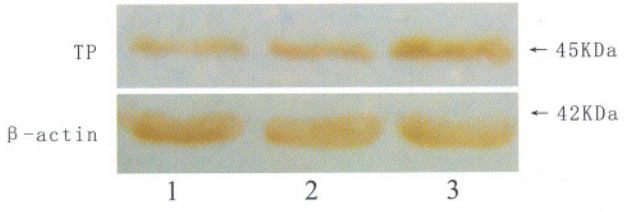

Figure 2. Results of IFN-alpha2b on the expression of TP protein Concentration of IFN-alpha2b (IU/ml). Lane 1: 0; Lane 2: 3,000; Lane 3: 6,000

3.3 IC_{50} of 5-FU in different treated group

In both 5-FU group and combination group, inhibition rate of treated 786-0 was calculated and is directly related with the concentration of 5-FU (Table 1). IC_{50} in 5-FU group is remarkably higher than that in combination group. The sensitivity of 786-0 cells to 5-FU was significantly enhanced in combination group($P < 0.05$).

Table 1. Inhibition rate and IC_{50} in 5-FU and combination group

5-FU (μmol /L)	5-FU		IFN + 5-FU	
	OD Value	IC_{50}(μM)	OD Value	IC_{50}(μM)
1×10^5	0.1748 ± 0.0056		0.1580 ± 0.0014	
1×10^4	0.2523 ± 0.0182		0.1898 ± 0.0046	
1×10^3	0.3203 ± 0.0221	13.9467 ± 3.7140	0.2283 ± 0.2165	5.3200 ± 0.1039
1×10^2	0.5000 ± 0.0248		0.3708 ± 0.0118	
10	1.1158 ± 0.0400		0.9930 ± 0.4429	
1	1.2623 ± 0.0405		1.1878 ± 0.4461	

3.4 Antitumor effect of different group *in vivo*

The weight of tumor were 1.1820 ± 0.4874 g in control group, 0.6900 ± 0.1517 g in 5-FU group and 0.2500 ± 0.1490 g in combination group. Treatment with 5-FU or 5-FU combined with IFN-alpha2b induced significant shrinkage of tumors compared with control group ($F = 11.533$, $P < 0.01$). Furthermore, combination treatment showed more effective than mere 5-FU treatment ($P < 0.05$). These results indicated that combination with IFN- alpha2b enhanced the sensitivity to 5-FU *in vivo*.

4 Discussion

5-FU is widely used in the treatment of cancers. However the response rate is only 5% – 20% in the treatment of renal cell carcinoma. Strategies have been explored to modulate the anticancer activity of 5-FU.

5-FU can be metabolized by two ways. One is DNA pathway, and the other is RNA pathway. Compared with RNA pathway, DNA pathway is more efficient in synthesizing fluorodeoxyuridine monophosphate (FdUMP) which is one of active metabolites. FdUMP, a critical metabolite of 5-FU, binds to the nucleotide-binding site of thymidylate synthase (TS). The combination inhibits TS, subsequently blocks forming thymine which tumors need to build their nucleic acids and leads to tumor cells apoptosis. TP proved to be the rate-limiting step of the straight activation of 5-FU to FdUMP. Increasing TP expression appeared to be the appropriate way to trigger the DNA pathway[3]. In our research it was observed that chemosensitivity of 786-0 cells to 5-FU was dramatically increased by combination IFN-alpha2b. The value of IC_{50} of 5-FU is 13.9467 μmol/L in sole use, while in combination the value decreased to 5.3200 μmol/L. In xenograft models better antitumor results were shown in combination group. Weight of tumor was merely 0.25 g in average under the treatment of combination, and under sole 5-FU treatment weight of tumor had reached 0.69 g. Those results proved combination can achieve better therapy effect. To clarify this result, our research detected the expression of TP and found expression of both TP mRNA and TP protein were up-regulated in 786-0 cells treated by IFN-alpha2b, which showed a dose-dependent manner with the IFN-alpha2b concentration. This finding indicated TP involved in enhanced chemosensitivity which was caused by combination use of 5-FU and IFN-alpha2b.

Although some pathological studies indicate TP had involved in angiogenic and antiapoptotic activities and correlated with unfavorable prognosis[4,5], it was highlighted that those studies had not accompanied with fluoropyrimidine drugs. In our study, no matter *in vitro* or *in vivo*, enhanced sensitivity to 5-FU was observed in 786-0 cells by upregulating TP which induced through combination therapy. Several studies discovered that other cytokines such as TNF- alpha or interleukin-1 increased TP expression in tumor cells and increased sensitivity to 5-FU. In xenograft models fluoropyrimidine drugs can be more efficient in inhibiting tumor growth by transfecting tumor cells with TP gene. Those experiments suggested fluoropyrimidine drugs can be particularly activated by TP and TP is an important molecular marker for reference when chemotherapy was adopted with fluoropyrimidine drugs[1,6]. In renal carci-

noma therapy, TP could be a candidate index to chemotherapy with fluoropyrimidine.

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