Effect of EGCG in combination with gemcitabine on β-catenin expression in PANC-1 human pancreatic cancer cells

Research Article

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Summary

Aim: To observe the effect of EGCG in combination with gemcitabine on PANC-1 human pancreatic cancer cells and to explore whether EGCG could enhance pancreatic cancer cell sensitivity to chemotherapeutic drugs by inhibiting β-catenin mRNA and protein through Wnt/β-catenin pathway. METHODS: Cultured PANC-1 cells were divided into control group, EGCG group, gemcitabine group (JX), and EGCG and gemcitabine group. Cells apoptosis was detected by flow cytometry. β-catenin expression was measured by RT-qPCR and Western blot. Results: JX + EGCG group had the strongest apoptosis, followed by EGCG group, JX group, and control group. β-catenin mRNA expression of JX + EGCG group was significantly lower than that of control group (P <0.05). β-catenin protein expression of JX + EGCG group was significantly lower than that of control group (P <0.01). Conclusion: Combination of EGCG and Gemcitabine had a synergistic effect on inducing PANC-1 apoptosis, possibly by inhibiting β-catenin mRNA and protein through Wnt/β-catenin pathway.
I. Introduction:
Research shows that, one of the important molecular characteristics of malignant tumor cell cycle is out of control, causing cell differentiation obstacles and growth disorders, leading to cell proliferation[1]. Literature suggested that Wnt/β-catenin pathway was related to tumor occurrence and development because it could regulate stem cell proliferation and differentiation [2]. Moreover, β-catenin pathway is a key hub for the whole pathway, its transfer from cytoplasm to the nucleus is considered to be the hallmarks of activation and function of this pathway [3]. Pancreatic cancer is one of the malignant tumors of the digestive system with very poor prognosis. It is easy to transfer and has low sensitivity to chemotherapeutic drugs and high levels of drug resistance. The number of new cases per year and the number of deaths per year are almost the same [4]. Epigallocatechin-3-gallate, EGCG is the main active ingredient of green tea extract polyphenols, and has broad anti-tumor effects [5]. Gemcitabine is a candidate for the first choice drug in the treatment of pancreatic cancer [6]. But the combination of the two is rarely reported. By using flow cytometry, fluorescence-based quantitative real-time PCR (RT-qPCR), and Western blot, we studied the effect of EGCG in combination with Gemcitabine Hydrochloride on PANC-1 human pancreatic cancer cells, aimed to explore the possibility of induction of apoptosis in pancreatic cancer cells by inhibiting β-catenin mRNA and protein through Wnt/β-catenin pathway.

II. Material and methods
Material: Total RNA extraction kit was purchased from Generay company (Beijing); RevertAid First Strand cDNA synthesis Kit and Protein ladder were from Fermentas (Beijing); IQ SYBR Green Supermix, 30% Acrylamide/Bis solution, and Glycine were purchased from Bio-Rad (Beijing). PVDF membrane was from Millipore (Beijing). ECL Plus kit was purchased from Bi-Yun-Tian (Beijing). Tris base and SDS were from Biosharp (Beijing). Gemcitabine Hydrochloride was from Lilly France.

Cell culture and grouping: PANC-1 human pancreatic cancer cells were purchased from Shanghai Institute of Biochemistry and Cell Biology and were cultured in high-glucose DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin in 5% CO₂ at 37 °C. EGCG and Gemcitabine Hydrochloride were dissolved in 0.9% saline.

Cells were randomly divided into 4 groups: (1) control group (control): nothing added; (2) EGCG group (EGCG): EGCC, 60µg/ml; (3) gemcitabine group (JX): Gemcitabine Hydrochloride, 20µg/ml; (4) EGCG and gemcitabine group (JX + EGCG): EGCC, 60µg/ml; Gemcitabine Hydrochloride, 20µg/ml.

MTT method: (1) cell inoculation. (2) cell culture. (3) discard the original medium, sequentially adding drugs with different concentration gradient, each group has 5 holes. At 37 °C, 5% CO₂ saturated humidity incubator culture. (4) adding corresponding role of time, remove the 96 orifice plate. Each hole with 10 µL CCK-8 solution, cultured for 1 h. (5) 450nm wavelength, read absorbance of each well value (A value) by the enzyme mark instrument.

Flow cytometry: 6 × 10⁴ PANC-1 cells were seeded to each well of 6-well plates and were grown in 5% CO₂ at 37 °C for 12 hours. 12 hours later. Discard the original medium was discarded and new medium with different drugs at the concentrations mentioned above was added. After treatment, cells were Trypsinized, washed with cold PBS twice, and collected by centrifugation. 100µL of 1X annexin-binding buffer was used to re-suspend cell pellets. After adding 5µL of Annexin V and 1µL of PI (100µg/mL), the mix was incubated for 15 minutes. Additional 400µL of 1X annexin-binding buffer was added before reading on the machine.

RT-qPCR: Total RNA was extracted from PANG-1 cells using Trizol reagent and was reverse transcribed into cDNA. Primers used for RT-qPCR were from Shanghai Sangon Biological Engineering Technology & Services Co. (Shanghai, China) (Table 1).
Western blot analysis: Following treatment, cells were collected with cell scrapers in ice-cold phosphate-buffered saline (PBS) followed by centrifugation (250 x g, 5 min). Cell extracts were prepared in RIPA buffer containing protease inhibitors (Calbiochem) with constant agitation at 4ºC for 30 min. After centrifugation at 15,000 x g for 20 min, protein extracts (30 μg) were resolved on 10% SDS-PAGE gels, transferred to PVDF membranes (BIO-RAD), and blocked with 5% non-fat milk as we described (33). PVDF membranes were incubated with primary antibodies (1: 1000 dilution) in TBS-T with 5% non-fat milk at 4ºC for overnight. After incubating with a peroxidase-conjugated secondary antibody, protein bands were visualized by ECL.

Statistical analysis. SPSS 13.0 was used for statistical analysis. χ² test or t test was used to do comparison between groups. P values < 0.05 was considered significant.

III. Results;

Inhibitory effect of EGCG combined with gemcitabine on proliferation of PANC-1 cells

MTT method results showed that Proliferation inhibition effect on PANC-1 cells of JX + EGCG group was higher than that in EGCG group and JX group (Table 2).

**EGCG in combination with gemcitabine induced PANC-1 cells apoptosis.**

Flow cytometry results showed that JX + EGCG group had the strongest apoptosis, followed by EGCG group, JX group, and control group (Table 3 and Figure 1).

**Effect of EGCG in combination with gemcitabine on β-catenin mRNA expression.**

Amplification curves for β-catenin gene were shown in Figure 2. The Ct values of the amplification curve were between 20 and 40, indicating that the amplifications were normal. The melting curves of β-catenin products showed one steep peaks (Figure 3), indicating there was no significant non-specific product.

RT-qPCR results showed that the β-catenin mRNA gene expression level of JX + EGCG group was significantly decreased when compared with that of control group (P <0.05) (Figure 4).
Effect of EGCG in combination with Gemcitabine on β-catenin protein.
Western blot analysis showed the β-catenin protein level of JX + EGCG group was significantly lower than that of control group (P <0.01) (Figure 5).

Table 3 Apoptotic effect of EGCG in combination with Gemcitabine Hydrochloride in PANC-1 cells

<table>
<thead>
<tr>
<th>group</th>
<th>control</th>
<th>JX</th>
<th>EGCG</th>
<th>JX+EGCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoptosis rate %</td>
<td>1.85±0.13</td>
<td>10.27±0.82*</td>
<td>17.06±0.62*</td>
<td>28.52±1.28*</td>
</tr>
</tbody>
</table>

*P<0.01 vs. control (48h);
□P<0.01 vs. JX (48h);
△P<0.01 vs. EGCG (48h).

Figure 1 Apoptosis of PANC-1 cells 48h post of treatment. *P<0.01 vs. Control group (48h); △P<0.01 vs. JX group (48h); ※P<0.01 vs. EGCG group (48h).
Figure 3: Melting curves of β-catenin.

Figure 4: β-catenin mRNA expression level of each group

Figure 5: Effect of EGCG in combination
Discussion

Recent studies show that EGCG and Gemcitabine Hydrochloride have anti-tumor effect both in vitro and in vivo. Tan M. et al [7] showed that EGCG could inhibit PANC-1 proliferation and might be used as an effective inhibitor of human pancreatic cancer. Studies from Shankar S. et al. [8] showed that EGCG inhibited the growth, invasion, metastasis of pancreatic cancer and angiogenesis, and thereby it might be used for the treatment and prevention of pancreatic cancer. Gemcitabine is currently used in the standard chemotherapy for pancreatic cancer [9]. Gemcitabine is a nucleoside analogs used in chemical therapy, mainly used in the clinical treatment of non small cell lung cancer, pancreatic cancer, etc., in patients with malignant tumor. Mitsunaga S. et al. [10] showed that gemcitabine could be used for treatment of advanced pancreatic cancer, and serum IL-6 and IL-1β levels could be used to predict the effect of gemcitabine on advanced pancreatic cancer. In this study, the cells were divided into 4 groups: (1) control group (control): nothing added; (2) EGCG group (EGCG): EGCC, 60ug/ml; (3) gemcitabine group (JX): Gemcitabine Hydrochloride, 20ug/ml; (4) EGCG and gemcitabine group (JX + EGCG): EGCC, 60ug/ml; Gemcitabine Hydrochloride, 20ug/ml. Flow cytometry results showed that experimental drugs could induce PANC-1 cells apoptosis compared with control group and the apoptosis-inducing effect was gemcitabine + EGCG > EGCG > gemcitabine which indicated that combination of EGCG and Gemcitabine Hydrochloride had a synergistic effect on inducing PANC-1 apoptosis.

It has been shown that it is the growth of very few cancer stem cells which have unlimited proliferative potential that causes the growth of tumor [11]. Recent studies [12] showed that Wnt/β-catenin pathway regulated stem cell proliferation and differentiation, and was related to tumor occurrence and development. β-catenin was the key molecule in Wnt / β-catenin signaling pathway and the transfer of β-catenin from cytoplasm to the nucleus was considered to be the hallmarks of activation and function of this pathway. In this study, to observe the effect of Gemcitabine Hydrochloride in combination with EGCG on the expression level of β-catenin mRNA, we measure the expression level of β-catenin mRNA from control group, EGCG group, JX group, and JX + EGCG group by using RT-qPCR. Results showed that the β-catenin mRNA expression level of JX + EGCG group was significantly lower than that of control group. We also analyzed the effect of Gemcitabine Hydrochloride in combination with EGCG on the expression level of β-catenin protein by Western blot analysis. The results showed that the β-catenin protein level of JX + EGCG group was significantly lower than that of control group, suggesting that combination of EGCG and Gemcitabine Hydrochloride could induce apoptosis in pancreatic cancer cells by inhibiting β-catenin expression through Wnt/β-catenin pathway.
References:


