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Effects of cyclopamine on the biological characteristics of human breast cancer MCF-7 cell line and its mechanism

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Summary

Purpose: To observe the effects of cyclopamine on the biological characteristics of human breast cancer MCF-7 cell line and explore its mechanism. *Materials and Methods:* After human breast cancer MCF-7 cells were treated with different-concentration cyclopamine for different periods, MTT assay was used to detect the inhibitory effect of cyclopamine on MCF-7 cell proliferation, flow cytometry was used to determine the distribution of MCF-7 cell cycle and the effect of cyclopamine on MCF-7 apoptosis, and Western blot was used to measure the protein levels of cyclins D1 and p21 in MCF-7 cells. *Results:* In certain range, MCF-7 cell proliferation was inhibited by cyclopamine in a dose- and time-dependent manner, and the optimal inhibiting concentration was ten μmol/L and the optimal action time at 48 hours. With the time prolongation of cyclopamine action, the cells in G_0/G_1 phase were significantly increased, but the cells in S phase were significantly decreased (compared with blank control group, all $p < 0.05$). With the time prolongation of cyclopamine action, apoptosis rate of MCF-7 cells was also significantly increased (compared with blank control group, all $p < 0.05$). The level of cyclin D1 of MCF-7 cells was decreased, but cyclin p21 was increased (compared with blank control group, all $p < 0.05$). *Conclusion:* Cyclopamine inhibits MCF-7 cell proliferation via arresting MCF-7 cell transformation from G_1 phase to S phase. This may be associated with the expressions of Hedgehog (Hh) signaling pathway-related cyclins.

Key words: Breast cancer; MCF-7; Cyclopamine; Cyclin.

Introduction

Breast cancer is a type of common malignancy and its formation is associated with the abnormal activation of Hedgehog (Hh) signaling pathway [1]. In this study, the authors observed the effects of cyclopamine, a Hh signaling pathway-specific inhibitor, on human breast cancer MCF-7 cells and explored the possible mechanism in order to provide a theoretical basis for clinical application of cyclopamine in the treatment of breast cancer.

Materials and Methods

All study methods were approved by ethics committee of the First Affiliated Hospital, Liaoning Medical University.

Cell culture

MCF-7 cells were incubated in RRPMI640 medium containing 10% of fresh fetal calf serum, 100 U/ml of penicillin and 100 U/ml streptomycin at 37°C at an atmosphere of 5% CO_2. Medium was changed every two or three days and a passage was performed every four to seven days.

MTT assay

MCF-7 cells (1×10^4/ml) in logarithmic growth phase were inoculated in a 96-well plate (each well with 100 μl of MCF-7 cells). When cells were adherent, the final concentrations (1, 2, 5, 10, and 15 μmol/L) of cyclopamine were respectively added into each well (each well with 100 μl of cyclopamine and each group with five wells) followed by incubation for 24, 48, and 72 hours, respectively. MTT (0.5 mg/ml, 100 μl) was added. Four hours later, 150 μl of DMSO was added with shaking. About 15 minutes later, the absorbance of each well was determined at 490 nm with ELLISA. Control group and blank control group were also set. The inhibition rate was calculated according to the following formula: inhibition rate = 1-absorbance in experiment group/absorbance in control group ×100%.

Flow cytometry

MCF-7 cells (1×10^6/ml) were collected after treated with ten μmol/L of cyclopamine for 24 and 48 hours respectively, and then partial cells underwent PI single staining for analysis of cell cycle and other cells underwent AnnexitinV-FITC/PI double staining for analysis of apoptosis.

Western blot

MCF-7 cells were inoculated in a six-well plate at 1.5×10^6/well overnight, and then treated with ten μmol/L of cyclopamine for 24 and 48 hours, respectively. MCF-7 cells underwent clearage on ice, degeneration in water bath, semi-dry transmembrane followed by addition of mouse anti-human monoclonal antibody of cyclins D1 and p21. The membrane was placed in alkaline phosphatase-labeled secondary antibody, and then visualized with luminous liquid prepared according to the manufacturer's instructions.

Statistical analysis

Statistical treatment was performed with SPSS16.0 software. Measurement data were expressed as (±*s*). Analysis of variance was used in experiment data and *q* test was used in the comparisons between groups. Statistical significance was established at $p < 0.05$.

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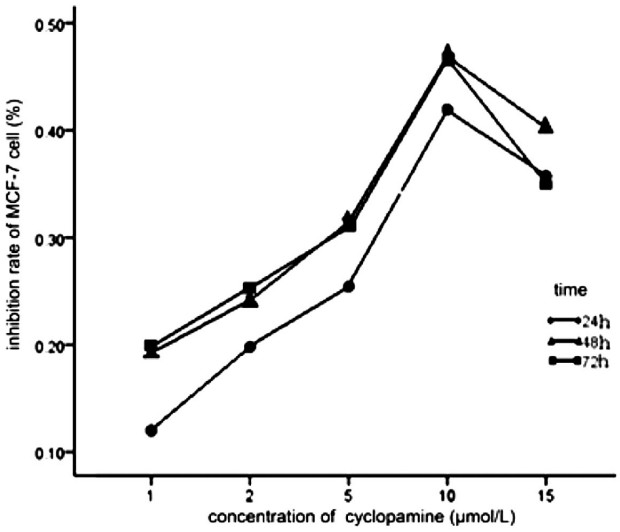

Figure 1. — Inhibition rates of MCF-7 cells caused by different-concentrations of cyclopamine.

Table 1. — *Inhibition rates of MCF-7 cells caused by cyclopamine (%).*

Groups	24 hours	48 hours	72 hours
1.0 μmol/l	11.99±6.70	19.31±2.86	19.89±2.98
2.0 μmol/l	19.76±3.36	24.06±2.41	25.32±1.78
5.0 μmol/l	25.41±3.04	31.43±3.38	31.04±3.52
10 μmol/l	41.92±1.32	46.95±4.12	46.62±3.28
15 μmol/l	35.72±3.70	40.20±2.66	35.02±3.39

Notes: There are significant differences between different-concentration groups at the same time point ($F = 157.16$, $p < 0.05$).
There are significant differences between different-time points at the same concentration ($F = 18.18$, $p < 0.05$).
There are significant differences between concentration and action time ($F = 1.29$, $p > 0.05$).

Results

Inhibitory effects of cyclopamine on MCF-7 cell proliferation

As shown in Figure 1 and Table 1, with the increases in the concentration and action time of cyclopamine, the inhibitory effects of cyclopamine on MCF-7 cell proliferation were not always increased, and the optimal inhibiting concentration was ten μmol/L and the optimal action time was 48 hours. There were significant differences in inhibition rate between different-concentration groups at the same time point and between different time points at the same concentration ($p < 0.05$). The concentrations were irrelevant to action time without interaction ($p > 0.05$).

Flow cytometry

With the time prolongation of cyclopamine action, MCF-7 cells in G_0/G_1 phase were increased, but MCF-7 cells in S phase were decreased (Figure 2). Compared with blank control group, more MCF-7 cells were arrested in G_1 phase in cyclopamine groups, suggesting that cyclopamine action was associated with MCF-7 cell transformation from G_1 phase to S phase, namely that the inhibition point of cyclopamine was G_1/S. With the time prolongation of cyclopamine action, MCF-7 cell apoptosis was increased. Compared blank control group, apoptosis rates in 24- and 48-hour-treated groups were significantly increased (all $p < 0.05$) (Figure 3).

Western blot

Effects of cyclopamine on the expressions of cyclins D_1 and p21 are shown in Figure 4. Compared with blank control group, cyclin D_1 expression was significantly decreased in cyclopamine groups ($p < 0.05$). Cyclin D_1 expression in 24- hour-treated group was (34.30 ± 0.79) % of cyclin D_1 expression in blank control group and in

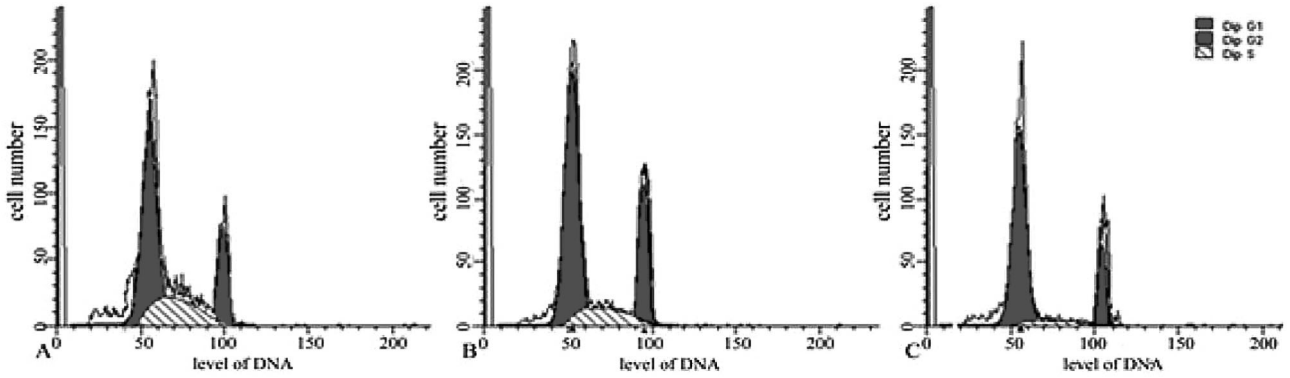

Figure 2. — Effects of cyclopamine on MCF-7 cell cycle determined with flow cytometer. A) Blank control group: 45.42% of MCF-7 cells in G_1 phase, 43.23% of MCF-7 cells in S phase, and 11.35% of MCF-7 cells in G_2 phase. B) Cyclopamine (24-hour-treated) group: 67.32% of MCF-7 cells in G_1 phase, 17.22% of MCF-7 cells in S phase, and 15.46% of MCF-7 cells in G_2 phase. C: Cyclopamine (48-hour-treated) group: 80.85% of MCF-7 cells in G_1 phase, 9.01% of MCF-7 cells in S phase, and 10.14% of MCF-7 cells in G_2 phase.

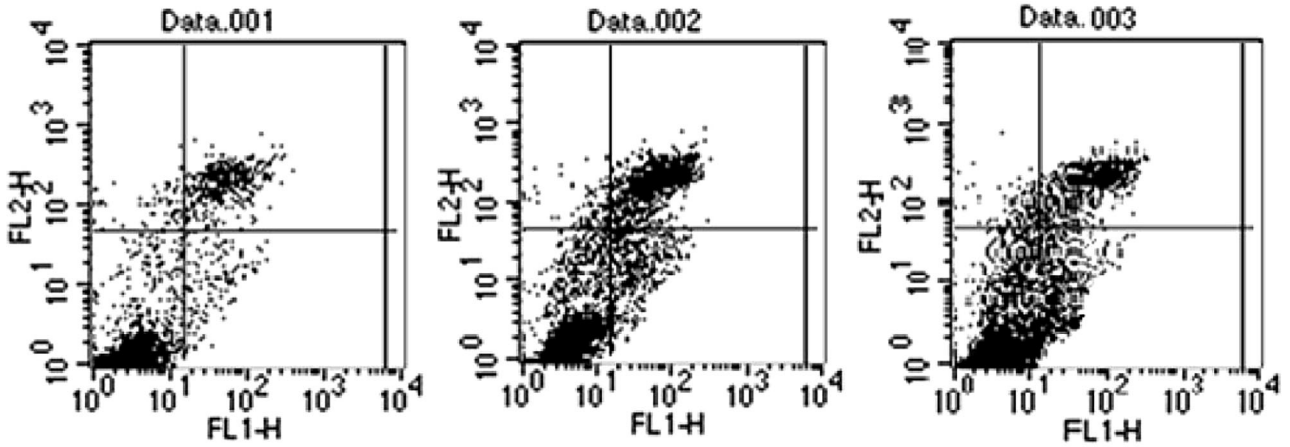

Figure 3. — Effects of cyclopamine on MCF-7 cell apoptosis determined with flow cytometer. A) Blank control group: apoptosis rate is 3.03%. B) Cyclopamine (24-hour-treated) group: apoptosis rate is 9.65%. C) Cyclopamine (48-hour-treated) group: apoptosis rate is 14.98%.

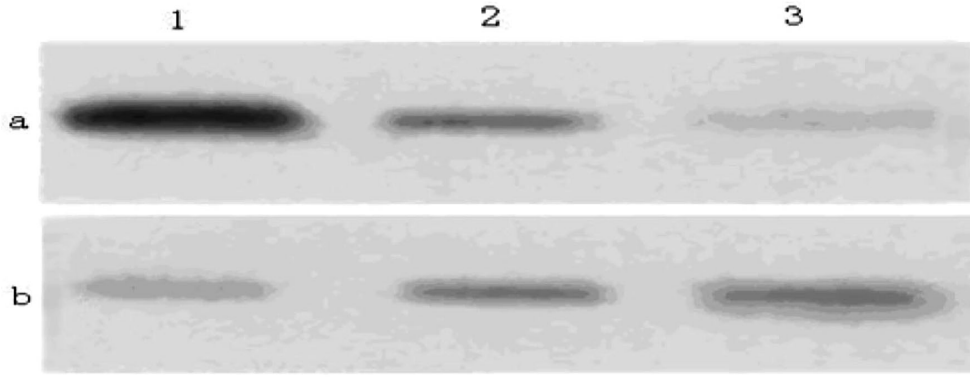

Figure 4. — Expressions of cyclins D_1 and p21 in each group detected with Western blot. Notes: Lane 1: blank control group, Lane 2: 24-hour-treated group and lane 3: 48-hour-treated group. a: cyclin D_1, b: cyclin p21.

48-hour-treated group was (21.46 ± 1.23) %. Compared with blank control group, cyclin p21 expression was significantly increased in cyclopamine groups (P<0.05). Cyclin p21 expression in 24-hour-treated group was (1.24 ± 0.02) times cyclin p21 expression in blank control group, and in 48-hour-treated group was (1.46 ± 0.02).

Discussion

Relationship between Hh signaling pathway and tumor cells

Hh gene is associated with Patched (Ptch) and smoothened (Smo) on the cell membrane. In the absence of Hh, Ptch, and Smo form a compound, inhibiting Smo activity. When Hh combines with Ptch, Ptch cannot inhibit Smo, hence Smo activity is released and the expressions of Smo signaling pathway-related downstream genes are up-regulated. Therefore, Hh signaling pathway-related molecular mutations can cause a variety of developmental defects or tumor [2, 3].

Relationship between cyclopamine and Hh signaling pathway

Cyclopamine, steroid alkaloids, and a kind of Hh signaling pathway-specific inhibitor, is extracted from the genus Veratrum. Cyclopamine can inhibit Hh signaling pathway activity [4]. It is reported that cyclopamine can inhibit tumor growth caused by the excessive activation of Hh signaling pathway and induce apoptosis [5].

Relationship between cyclins and Hh signaling pathway

The aberrant activation of Hh signaling pathway is related to tumorigenesis, and target genes or downstream molecules of Hh signaling pathway such as n2Myc, Egf, cylin D, cylin E, cylin B, and BMP are involved in tumor cell proliferation and invasion [6]. Cyclin D_1, an important member of G cyclin family, is regarded as an oncogene. Cyclin D_1 can activate CDK4 and CDK6 in G_1 phase and accelerate the transformation from G_1 phase to S phase [7]. p21, a cell cycle-regulatory factor, can inhibit cyclin D_1 activity and

lead to cell cycle arrest, interfering with cell proliferation and playing a negative regulatory role in cell proliferation [8].

Present study

In this study, after human breast cancer MCF-7 cells were treated with cyclopamine, MTT, flow cytometry, and Western blot were performed. This study suggests that cyclopamine can inhibit MCF-7 cell proliferation and induce MCF-7 cell apoptosis via downregulation of cyclin D_1 and up-regulation of cyclin p21. Whether there are other mechanisms regarding cyclopamine-induced breast cancer cell apoptosis remain to be further studied.

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