EFFECTS OF $p16^{INK4}$ GENE ON CHEMOSENSITIVITY OF HUMAN GLIOMA U251 CELL LINE TO TENIPOSIDE

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ABSTRACT

Objective: To determine the effects on the cell growth, tumorigenicity and chemosensitivity of p16/CDK4I in human glioma. Methods: p16 gene was transfected into U251 cells by lipofectin. Expression of exogenous p16 gene was confirmed by immunohistochemistry and Northern blot. The effects of exogenous p16 gene on the growth and chemosensitivity to teniposide were examined. Results: Expression of exogenous p16 gene inhibited the growth dramatically *in vitro*. G1 arrest of tumor cells was observed. However, wt p16-positive U251 was less sensitive than control cell lines and the number of apoptotic cells after chemotherapy was reduced. Conclusion: The expression of exogenous p16 gene could inhibit the growth of glioma. On the other hand, the chemosensitivity to teniposide of p16-positive U251 was decreased.

Key words: Glioma, p16 gene, Teniposide, Chemosensitivity

$P16^{INK4}$ is an important anti-oncogene discovered recently. It served as a negative cell cycle regulator which specifically binds to and inactivates cyclin-dependent kinase (CDK4). In a lot of primary tumors and tumor cell lines, $P16^{INK4}$ was found lost or mutated, especially in 71%-82% of human gliomas.[1,2] In our work, we transfected $P16^{INK4}$ into human glioma U251 cell line which has homozygous deletion of $P16^{INK4}$. It was found that $P16^{INK4}$ could decrease the growth rate of U251, but on the other hand, the chemosensitivity to teniposide was decreased. The inhibition of apoptosis induced by teniposide (VM26) must be the main mechanism.

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MATERIALS AND METHODS

Chemicals

Newborn calf serum, lipofectin and DMEM were the products of GIBCO-BRL, geneticin (G418) was the product of Amersham, MTT was bought from SIGMA, DMSO was the product of Farco, a mouse monoclonal antibody (IgG) against human p16 was from Wuhan BOSTER Company, ABC immunohistochemistry kit was from VECTOR, DIG labeling and detection kit was the product of Boehringer Mannheim.

Plasmids

Human $P16^{INK4}$ plasmid of pCDNA3-neo-p16 was presented by Kagawa Medical University of Japan. Plasmid of pCDNA3 was preserved in our laboratory.

Cell Culture

Human glioma U251 cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated newborn calf serum at 37°C under 5% CO_2 in a humidified atmosphere. Cell viability was evaluated by way of trypan blue dye exclusion.

The Transfection and Selection of U251

Human glioma U251 cells were transfected by lipofectin and selected by 500 μg/ml G418 for 8 weeks. At the same time, control U251 cells were transfected with pCDNA3 plasmid.

Northern Blotting Analysis

Cell line RNAs were isolated and electrophoresed through formaldehyde-agarose gels (1.0%), and blot-transfected and fixed to a nylon-based nitrocellulose

membrane by baking in a vacuum oven at 80°C for 2 h. Then, $P16^{INK4}$ probe (560 bp, HindIII, XhoI fragment from the plasmid) was labelled and the filter was hybridized and dedected under the direction of the manual of the kit.

Measure of Cells Growth

Cells growth curves were drawn by the methods of MTT colorimetric assay which was descripted previously.[3]

Chemosensitivity Assay

Every cell line was cultured into 96-well microtiter plate at a density of 10000/200μl/well separately. After 24 h, the culture medium was pipetted off and teniposide was added to wells by doubling dilution, while the control wells were added culture medium of the same volume but without drugs. After 48 h, 20 μl MTT (5 mg/ml in 0.01mol/L PBS) was added to every well, then oscillated the plate for 10 min and the OD value of each well was measured at the wavelength of 570 nm. The inhibitory rates of drugs were calculated by the formula[4]:

$$\frac{\text{OD value of control wells} - \text{OD value of experimental wells}}{\text{OD value of control wells}} \times 100\%$$

Flow Cytometry Assay

Flow cytometry assay of every experimental group was done by the method which was descripted previously by I. Nicoletti.[5] The 200 g centrifuged cell pellet was gently resuspended in 1.5 ml hypotonic fluorochrome solution (PI 50 μl/ml in 0.1% sodium citrate plus 0.1% Triton X-100), in 12×75 polypropylene tubes. The tubes were placed at 4°C in the dark overnight before the flow-cytometric analysis.

RESULTS

p16 Expression of Cells after Transfection and Selection

U251 cells after transfection of p16 (U251-p16) exhibited p16 positive immunoreactivity by immuno-histochemical analysis while control cells were negative (Figure 1). Northern blotting analysis showed that the mRNA level of p16 gene of U251-p16 was much higher than control cells (Figure 2).

The Inhibition Effects of p16 Gene on U251 Glioma Cells

Growth curves of the cells showed that the growth rate of glioma cells was decreased evidently after p16 transfection (Figure 3). FCM assay demonstrated that Gl arrest of glioma cells had happened (Figure 5).

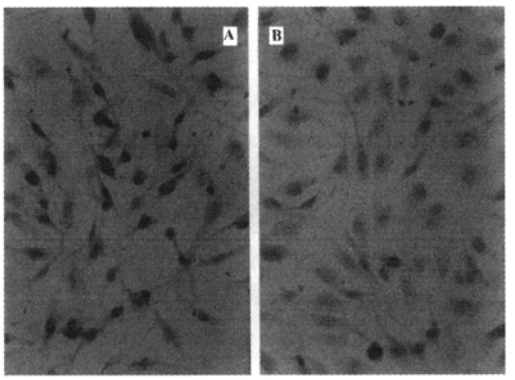

Fig. 1. Immunohistochemistry assay of expression of p16 (IH×100)
A. Positive expression of p16 on p16 transduced U251
B. Negative expression of p16 on pCDNA3 transduced U251

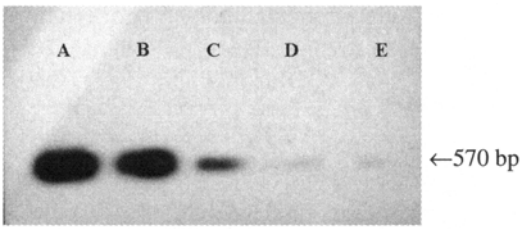

Fig. 2. Northern blotting of expression of p16 gene
A. U251-p16; B. U251-p16; C. U251 after 48 h by transient transfection of p16; D. U251-PCDNA3; E. U251

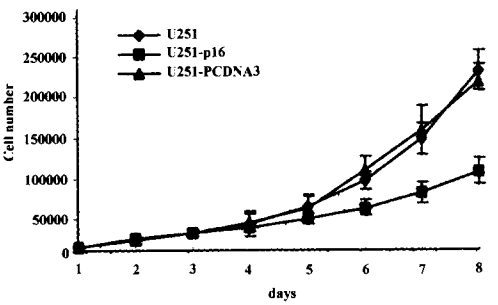

Fig. 3. Effects of expression of p16 on the growth rate of U251

The Effects of p16 Gene on Chemosensitivity of U251

After transfection of p16 gene, inhibitory rate of teniposide were decreased (Figure 4). FCM assay showed

that the diploid peak of U251-p16 after the treatment of teniposide became smaller and lower dramatically (Figure 5).

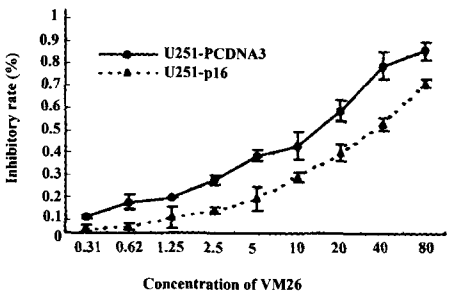

Fig. 4. Effect of expression of p16 on VM26 sensitivity of U251

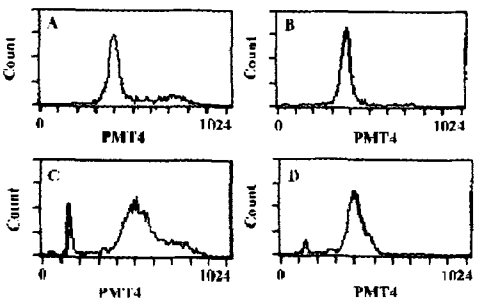

Fig. 5. Comparison of flow cytometric DNA fluorescence profiles of U251 cells (PI fluorescence)

A. Flow cytometric DNA fluorescence profiles of U251

B. Flow cytometric DNA fluorescence profiles of p16 transduced U251 -p16

C. Flow cytojmetric DNA fluorescence profiles of U251 after treatment of VM26 (2.5 μg/ml)

D. Flow cytometric DNA fluorescence profiles of U251-p16 after treatment of VM26 (2.5 μg/ml)

DISCUSSION

P16 gene is the most frequently deleted gene in gliomas. Among glioma cell lines, the deletion rate of p16 gene is found to be 81% and it is 71% in primary gliomas.[1,2] These results indicate that p16 deletion is involved in glioma formation and progress. At present, the biochemical properties of p16 indicate that it could be a negative regulator of the proliferation of normal cells. It binds with cyclin-dependent kinase 4 (CDK4) and inhibits the activity of CDK4-cyclin D enzymes. p16 gene seems to act in a regulatory feedback circuit with CDK4, D-type cyclins and the retinoblastoma protein.[6] Our results also demonstrated p16 gene could inhibit glioma cell growth.

The relationship between p16 gene and chemosensitivity of glioma cells is rarely reported in the present. We found that, after p16 gene transfection, the chemosensitivity of U251 glioma cells to teniposide was decreased evidently and flow cytometry assay showed that the apoptosis induced by teniposide was inhibited. This is an interesting phenomenon. The mechanism may relate to that: p16 gene can induce Gl arrest of glioma cells. As the result, the number of glioma cells in phase S, G2, M was reduced. So, because the target phases are phase S and G2, teniposide can hardly induce apoptosis of glioma cells and the chemosensitivity is decreased. At the same time, because of Gl arrest of glioma cells, this can help glioma cells to repair DNA damage. These two effects lead to that U25l glioma cells is less sensitive to teniposide. Recently, Datta and Kaufmann reported that, VP-16 and CDDP could activate a series of caspase enzymes.[7, 8] Besides that, VM26 can increase the expression of FasL and induce the apoptosis of tumor cells.[9] However, whether p16 gene could affect these pathways is scarcely known by now.

Because of the advantages of p16 gene, it has been a good target gene for genetherapy of glioma. However, before a radical treatment for tumors is found, the therapy of tumors must be multiple methods combined with operation, chemotherapy, radiotherapy, biotherapy and so on. As the result, when we do some research on one treatment factor, we must know whether and how it can act on other methods of treatment at the same time. Only after we have known all of that, can we get the best curative effect of tumors.

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cytidines were not transformed to uridines and the unmethylated cytidines were transformed after sodium bisulfite modification.[3] Therefore, the tPCR product of the methylated hMLH1 promoter must be sensitive to BstUI cleaving and that of the unmethylated one must be resistant to. We observed that the tPCR product from RKO was sensitive to BstUI cleaving, but the tPCR product from PACM82 was resistant. These results indicated that the hMLH1promoter of RKO cells was methylated as previously reported [4] and that of PACM82 cells was unmethylated.

Only one strong peak at the retention time 5.3 min could be detected in all the denatured testing wPCR products and the mixture of PACM82 and RKO cells by DHPLC at their partial denaturing temperature 62°C. The phenomenon indicates that all the testing wPCR products have the same sequence. The hMLH1 promoter of RKO cell is wild-type.[5] Therefore, the hMLH1 promoter of the testing gastric carcinoma specimen and PACM82 cell line must be wild-type.

The tPCR products of PACM82 and RKO cells were separated at 53°C completely (retention time, 6.2 min and 6.7 min, respectively). Two peaks were observed on the chromatogram of the denatured tPCR mixture of these cell lines (Figure 1A). The four-peaks typical mutation chromatogram was also obtained from the tPCR products of the testing gastric carcinoma specimen (Figure 1B). This result suggests that the tumor tissue contains both the methylated and the unmethylated hMLH1.

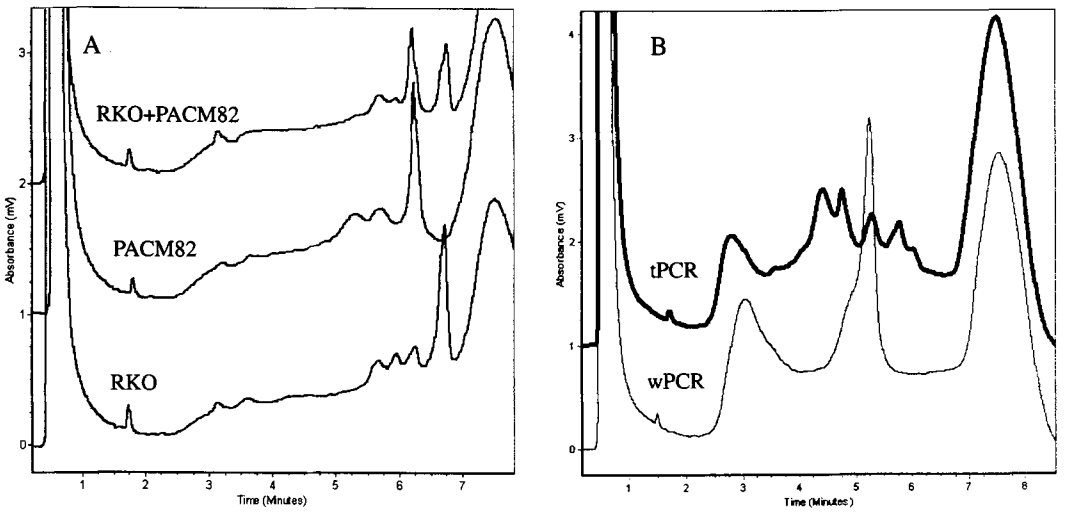

Fig. 1. DHPLC chromatograms of denatured PCR products of the hMLH1 promoter. HMLH1 promoters were amplified by tPCR or wPCR after bisulfite modification or without modification, respectively. The PCR products were partial denatured at 53°C for tPCR or 62°C for wPCR, and separated by DHPLC. A: tPCR products of RKO cells, PACM82 cells, and their tPCR mixture; B: tPCR and wPCR products of gastric carcinoma specimen

In DHPLC analysis, the PCR-amplified region contains several CpG sites, and methylation at any CpG site will lead to the delay of the PCR product. Thus, this analysis can analyze methylation at multiple CpG sites in the promoter region, showing the advantages over the previously existent methods for methylation analysis, such as methylation sensitive enzyme digestion, methylation specific PCR (MSP), etc. In conclusion, the methylated and the unmethylated tPCR products of the hMLH1 promoter could be separated by DHPLC conveniently.

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