Establishment and characterization of DNA pol β knockout human esophageal carcinoma cell line EC9706

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Abstract- To construct a DNA polymerase β gene knockout model in human esophageal carcinoma cell EC9706 by homologous recombination for investigating its biological characterization and sensitivity upon damaging factors or chemotherapeutics. Methods: Based on the homologous recombination principle, the gene targeting vector was constructed to delete polβ gene. The vector was introduced into esophageal carcinoma cellline EC9706 by electroporation. PCR, RT-PCR and Western blot were used to detect the expression of polβ gene at DNA, mRNA and protein level in polβ knockout EC9706 cell. Flow cytometry and MTT were used to detect cell cycle and cell growth velocity. The sensitivity of the gene targeting cell line upon oxydizing agent and chemotherapeutics were detected by trypan blue anti-dyeing method. Results: In the targeting cell line, the DNA, mRNA and protein expression of polβ can not be detected and its biological characterization has marked disparation compared with the normal EC9706 cell.Conclusion: The polβ gene knockout EC9706 cell line was constructed successfully. It may lay a foundation for the further study of pol β gene. [Life Science Journal. 2010; 7(2): 13 – 18] (ISSN: 1097 – 8135).

Key Words: DNA polymerase β; gene knockout; human esophageal carcinoma.

1. Introduction

DNA polymerase β (pol β) is a key enzyme in base excision repair (BER). Its main function is to make up the short gaps generated by base excision (1). Polβ may also take part in DNA duplication, recombination, genome stability and drug resistance (2-6). Furthermore, polβ is responsible to oxidative damage (7). The vicious pol β may decrease the ability of base excision repair and increase the hypersensitity to some alkylating agents (MMS) and oxidant (H_2O_2) (8). Studies showed that there exist overexpression of polβ in many kinds of tumor cells and it may be relevant to tumor's generation (9-10). Recently, polβ mutation and abnormal expression have been found in many human carcinomas (11), such as gastric cancer (12), bladder carcinoma (13), and prostate carcinoma (14-15). Therefore, further researches of polβ on expression characteristic in tumors have been a hotspot (16).

Gene targeting via homologous recombination is a powerful means of assessing gene function in vivo (17). It has been applied to diverse organisms such as bacteria, yeast, poultry and rodents (18). In addition, human somatic cell gene targeting has also been completed successfully (19). Compared with antisense nucleotide and siRNA, gene targeting can delete aimed gene completely. In order to better understand the function of DNA polβ in esophageal carcinoma, we plan to construct the gene targeting model of polβ.

2. Materials and methods

2.1 Cell line

Human esophageal carcinoma cell line EC9706 was purchased from state key laboratory of molecular oncology of Chinese Academy of Medical Sciences and cultured in RPMI-1640 medium containing 100g/L fetal bovine serum, at 37 ℃ in a 5% CO_2 humidified atmosphere. It was established in 2002, and separated from the well differentiated esophageal squamous carcinoma tissue of a Chinese male patient (20).

2.2 Construction of targeting vector

The targeting vector was created by cloning strategy. The upstream (1.2kbp) and downstream (1.8kbp) homologous sequences were obtained from the genome of esophageal carcinoma EC9706 cell by PCR. The two fragments were cloned into pcDNA3.1 which contains a neomycin resistance cassette for G418 selection. PCR and restriction enzymes digestion were used to identify the positive recombinant (pOUT-polβ). Then the targeting vector pOUT-polβ was linearized with EcoR V. 2.3 2.3 Electroporation 2×107 cells/ml EC9706 cells suspended in PBS (phosphate buffered saline) and 50μg lineared plasmids were prepared. Electroporation was performed on the Gene Pulser (Bio-Rad, Hercules, CA, USA) in 0.4cm cuvettes (Bio-Rad) at the following conditions: 250 Volts, 1000μF, 7s, ∞resistance. Then the cells were planted on 10cm culture capsule, 24 hours later the medium were replaced by RPMI-1640 with 800μg/ml G418. The positive monoclone was picked for expanded culture in RPMI-1640 with 200μg/ml G418.

2.3 PCR analysis

Total DNA were isolated from the gene targeting cells and the normal EC9706 cells (employed as the control group) using DNA extraction kit according to the

manufacturer's protocol (Qiagen, USA) respectively. The primers for polβ (5'AAAGGATTCCAGATAAACAC 3' , 5' GCTGGAAGGA AAGAAGAAAG 3') were used to identify whether polβ gene had been knocked out. PCR conditions were: initial denaturation at 94°C for 3 min, 35 cycles of amplification (94°C for 45s, 55°C for 45s, 72°C for 45 s), final extension at 72°C for 2 min, and then cooling to 4°C. The PCR products were separated on 15g/L agarose in 1×TAE and visualized by ethidium bromide staining.

2.4 RT-PCR analysis

Total cellular RNA was extracted by Trizol (Invitrogen, Carlsbad, CA). Reverse transcription was performed by AMV (Promega, USA).PCR was performed with DNA pol β and β actin (as ento-standard) specific primers, and the products were analyzed with 15g/L agarose gel electrophoresis. The primers were as follows: polβ (5'GAGAAGAACGTGAGCCAAGC3', 5'CATCCATGTCACCACTGGAC3'), β-actin (5'ACACTGTGCCCATCTACGAGG3', 5'CTTTG CGGATG TCCACGTC3').

2.5 Western blot analysis

Cells were washed with cold PBS, harvested and resuspended at a cell density of 106 cells/20μl in buffer I (10mM Tris-Cl, PH7.8, and 200mM KCl). An equal volume of buffer II (10mM Tris-Cl, PH7.8, and 200mM KCl, 2mM EDTA, 40%glycerol, 0.2% Nonidet P-40, 2mM dithiothreitol, 0.5mM phenylmethylsulfonyl fluoride, 10μg/ml aprotinin, 5μg/ml leupeptin, 1μg/ml pepstain) was added. Then, the cell suspension were transferred to microcentrifuge tubes and sonicated for 10s. After centrifugation of the sonicated suspension at 10000×g for 10 min, at 4 ℃, the supernatant was collected. The protein concentration was determined by coomassie brilliant blue. Equivalent amounts of protein (25 μg) were separated by SDS-PAGE and then transferred to polyvinylidene difluoride membranes. Membranes were then incubated for 1 h at room temperature with the blocking reagent [5% milk, 2% BSA, 40 ml Tris-buffered saline-0.5% Tween-20 (TBST) pH 7.6], and then incubated overnight at 4℃with the primary antibody. The membranes were washed in TBST and incubated with anti-rabbit secondary antibody for 45 min at room temperature. After washing the membranes with TBST, they were visualized by the protein a peroxidase-linked process (Amersham Biosciences, Little Chalfont, and UK).

2.6 Cytotoxicity assay

Cells were seeded into 24-well plates (1×105 cells/well).48 hr later when they were approximately 90% confluent, the cells were treated with different concentration of cisplatin, bleomycin, H2O2 and methylene blue respectively(Doses are shown in Table.1).2 days later the death rates were detected by trypan blue anti-dyeing method.

2.7 Cell Proliferation Assay

5×103 cells in logarithmic growth phase were seeded in 96-well plates (200μl/well) and allowed to grow for 7 days. Each group made seven blanket wells. Absorbance was measured at 490nm everyday. Four hours before stop culturing, 20 μL of 5 mg/mL MTT (Sigma) was added to the culture medium. After incubation, the culture medium was removed and 200 μL of dimethylsulphoxide (DMSO) was added to resolve the crystal. The cell proliferation curves were drawn according to the absorbance.

2.8 Flow Cytometry Assay

Blood-serum starvation method was used to synchronize cells in G0 phase. A total of 1×106 cells were trypsinized and washed with PBS twice. Then cells were fixed with prechilled 75% ethanol at 4℃ overnight. After three times of wash, cells were digested with 0.1% RNase at 37℃ for 20 min and stained with 10 μg/mL propidium iodide (PI, Sigma) for 1 hr at 4℃. Samples were assayed by flow cytometer and data were analyzed at 488nm.

2.9 Statistical Analysis

All data were analyzed with SPSS 13.0 Software, and conducted with single factor variance analysis (ANOVA). P-values<0.05 were considered statistically significant.

3. Results

3.1 Existence and expression of polβ gene in targeting cell

The existence, mRNA and protein expression of polβgene in the targeting cells and normal untreated EC9706 cells were detected by PCR, RT-PCR and western-blot respectively. The PCR result showed that in the control group (normal EC9706) there was a marked band located in 499bp but there is no existence of polβin the targeting cell (Figure 1). RT-PCR and Western-blot both revealed that there was no expression of polβin the polβknockout cells (Figure 2, Figure 3).

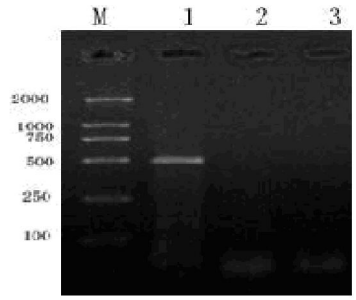

Figure 1. Agarose Gel Electrophoresis of PCR Products

Extract both of the normal EC9706 cells and the targeting cells genomes, then amplified by PCR. The results showed that in the control group (normal EC9706) there was a marked band located in 499bp but there is no existence of polβin the targeting cells. M: DNA Marker; lane1: Control cells (EC9706); lane 2 and 3: polβ knockout EC9706 cells

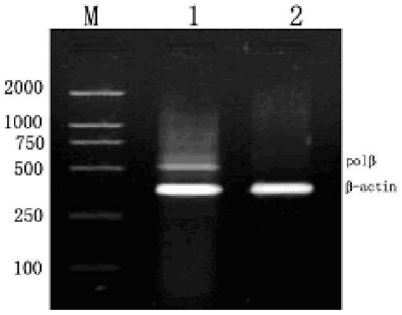

Figure 2. Agarose Gel Electrophoresis of RT-PCR Products

Extract both of the normal EC9706 cells and the targeting cells mRNA, and then amplified by PCR. There was no expression of polβ in the polβknockout cells. M: Marker; lane1: Control cells (EC9706); lane2: polβknockout EC9706 cells

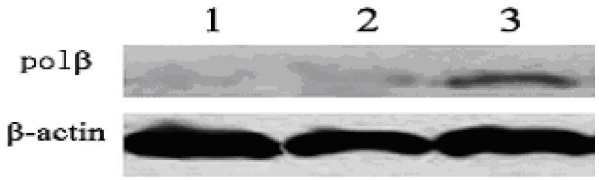

Figure 3. Western Blot Result

The whole cells proteins were prepared as described above in material and method, and samples (25 μg) subjected to Western blotting with polβ (SC-48819, Santa Cruz Biotechnology) and β-actin (SC-130656, Santa Cruz Biotechnology) antibodies. Lane1 and 2: polβ knockout EC9706 cells, there was no expression of polβ in the polβknockout cells; 3: Control cells

3.2 Cell sensitivity to DNA-damaging agents

Trypan blue anti-dyeing method was used to observe the sensitivity to DNA-damaging agents such as cisplatin, bleomycin, H2O2 and methylene blue of targeting cells and control cells. As shown in Figure4(A),4(B),4(C), beginning from the primary concentration, the death rates of the targeting cells to cisplatin, bleomycin and methylene blue were higher than that of control cells($P<0.05$). It means that the sensitivities of the polβ knockout EC9706 cell to these drugs were increased. In sensitivity to H2O2, there is no difference between the two groups when the concentration of H2O2 is below 160μmol/L ($P>0.05$). See in Figure4 (D). While the concentration of H2O2 is higher than 160μmol/L, the sensitivity of the polβ knockout EC9706 cell was increased.

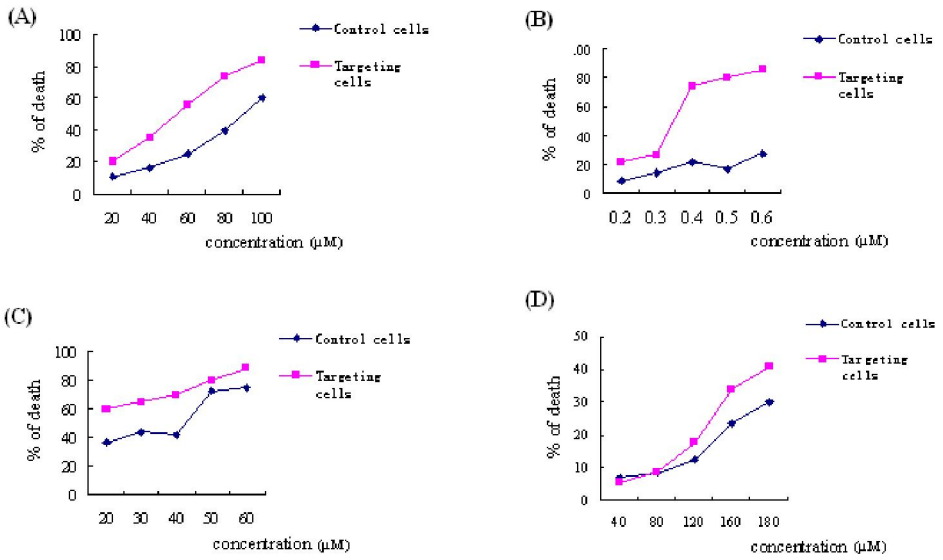

Fig.4 Cell sensitivity to DNA-damaging agents

3.3 biological behaviour of targeting cells

3.3.1 Cell growth curve

Proliferation of targeting cells was evaluated using the MTT assay (Figure.5). The absorbance-time curves showed that the growth velocity of targeting cells was significantly lower than that of control cells.

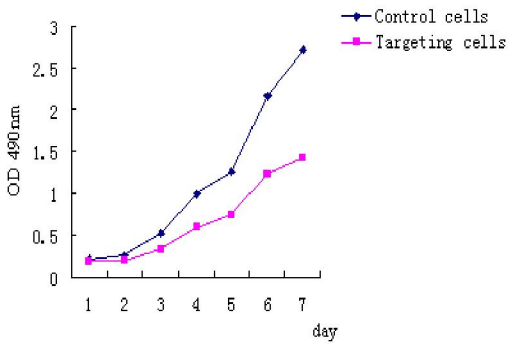

Fig.5 Proliferation curve of targeting cells and control cells

The absorbance-time curves showed that the growth velocity of targeting cells was significantly lower than that of control cells.

Trypan blue anti-dyeing method was used to observe the sensitivity to DNA-damaging agents of targeting cells and control cells. (A) Cisplatin (B) Bleomycin (C) Methylene blue (D) H2O2. As shown in Figure4(A), 4(B) and 4(C) were beginning from the primary concentration, the death rates of the targeting cells to cisplatin, bleomycin and methylene blue were higher than that of control cells ($P<0.05$). Figure4 (D) The sensitivity to H2O2, there is no difference between the two groups when the concentration of H2O2 is below 160μmol/L ($P>0.05$). While the concentration of H2O2 is higher than 160μmol/L, the sensitivity of the polβ knockout EC9706 cell was increased.

Table 1. Concentration of drugs

Drug	Final concentration（μmol/L）				
cisplatin	20	40	60	80	100
bleomycin	0.2	0.3	0.4	0.5	0.6
methylene blue	20	30	40	50	60
H2O2	40	60	80	160	200

3.3.2 Cell cycle

The cell cycle distribution of targeting cells and control cells was shown in Table 2. There have significant differences between targeting cells and control cells in G2~M phase and S phase ($P<0.05$). While no differences in G0-G1 phase.

Table.2 Cell cycle $\overline{}$（%,x±s）

Group	G0～G1(%)	S (%)	G2～M（%）
targeting cells	54.877±6.374	35.700±9.43	9.426±4.206
Control cells	46.673±1.616	53.403±1.504	0

4. Discussion

4.1 Construction of DNA pol β gene targeting vector

Based on homologous recombination principle, gene targeting was performed on many organisms which aim to exchange the gene between recipient cells' and the exotic gene, set the interesting gene at right site and alter the cells' emphytic character (21). As it was affected by many factors, design and construct of gene targeting vector will be the main points for gene targeting (22). In this research, we meant to replace part homologous sequences of polβ gene, finally, destroy the biological function of DNA polβ. The whole sequence was about 7.8kb (the upstream is 1280bp and the downstream is 1756bp). If occured, polβ gene core promoter will be replaced, as well as its first, second and third exons, first and second introns and bulk of the third intron. Finally, it may lead to loss of polβ gene function.

4.2 Influence on targeting cell cycle and proliferation

The cell cycle and proliferation rate of targeting cells changed compared with that of the control group (EC9706). For the targeting cells, the S phase rate decreased obviously and the proliferation was also step down ($P<0.05$). According to these results, we found that the proliferation of polβ gene targeting cells were affected, mainly displayed depressant effect. However, overexpression of polβ gene may lead to continuous malignant transformation and accelerated growth velocity (23). Therefore, polβ gene targeting can slow down tumor cell proliferation which will apply for the biotherapy of human carcinoma.

4.3 Cell sensitivity to DNA-damaging agents

The main function of polβ is base excision repair (BER) which is the major repair system for oxidative (H2O2, methylene blue) as well as alkylation damage (24). At present, induction of polβ while responding to oxidative stress has been shown in cultured cells and in organisms (25-26). In our research, polβ gene targeting cells were more sensitive to cisplatin, bleomycin and methylene blue, compared with control cell (EC9706). According to reference reports (27), polβ knockdown cells performed by RNAi technique were more sensitive to cisplatin, methylene blue and H2O2; the sensitivity to cisplatin, methylene blue was the same as that of polβ targeting cells while bleomycin was absolutely different. That may due to different expression level of polβ (polβwas deleted completely in targeting cells while there is very low level expression of polβin knockdown cells).

In conclusion, we constructed polβ gene targeting model in human esophageal carcinoma cellliwash basin (EC9706) and the deleting of polβ gene from background could remove the interference on experimental data. It provides important data for the further study of DNA polβ mutation and abnormal expression in human esophageal carcinoma.

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