

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 pol β gene. The vector was introduced into esophageal carcinoma cell line EC9706 by electroporation. PCR, RT-PCR and Western blot were used to detect the expression of pol β gene at DNA, mRNA and protein level in pol β 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 oxidizing agent and chemotherapeutics were detected by trypan blue anti-dyeing method. Results: In the targeting cell line, the DNA, mRNA and protein expression of pol β can not be detected and its biological characterization has marked disparation compared with the normal EC9706 cell. Conclusion: The pol β 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). Pol β may also take part in DNA duplication, recombination, genome stability and drug resistance (2-6). Furthermore, pol β is responsible to oxidative damage (7). The vicious pol β may decrease the ability of base excision repair and increase the hypersensitivity to some alkylating agents (MMS) and oxidant (H_2O_2) (8). Studies showed that there exist overexpression of pol β in many kinds of tumor cells and it may be relevant to tumor's generation (9-10). Recently, pol β 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 pol β 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 pol β in esophageal carcinoma, we plan to construct the gene targeting model of pol β .

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 °C in a 5% CO₂ 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-pol β). Then the targeting vector pOUT-pol β was linearized with EcoR V. 2.3 2.3 Electroporation 2 \times 10⁷ cells/ml EC9706 cells suspended in PBS (phosphate buffered saline) and 50 μ g 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, 1000 μ F, 7s, ∞ resistance. Then the cells were planted on 10cm culture capsule, 24 hours later the medium were replaced by RPMI-1640 with 800 μ g/ml G418. The positive monoclonal was picked for expanded culture in RPMI-1640 with 200 μ g/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 pol β (5'AAAGGATTCCAGATAAACAC 3' , 5'

GCTGGAAGGA AAGAAGAAAG 3') were used to identify whether pol β 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: pol β (5'GAGAAGAACGTGAGCCAAGC3', 5'CATCCATGTCACTACTGGAC3'), β -actin (5'ACACTGTGCCCATCTACGAGG3', 5'CTTTG CCGATG TCCACGTC3').

2.5 Western blot analysis

Cells were washed with cold PBS, harvested and resuspended at a cell density of 106 cells/20 μ l 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, 10 μ g/ml aprotinin, 5 μ g/ml leupeptin, 1 μ g/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 \times g for 10 min, at 4°C, the supernatant was collected. The protein concentration was determined by coomassie brilliant blue. Equivalent amounts of protein (25 μ g) 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°C 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 \times 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 \times 10³ cells in logarithmic growth phase were seeded in 96-well plates (200 μ l/well) and allowed to grow for 7 days. Each group made seven blank wells. Absorbance was measured at 490nm everyday. Four hours before stop culturing, 20 μ L of 5 mg/mL MTT (Sigma) was added to the culture medium. After incubation, the culture medium was removed and 200 μ L 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 \times 10⁶ cells were trypsinized and washed with PBS twice. Then cells were fixed with prechilled 75% ethanol at 4°C overnight. After three times of wash, cells were digested with 0.1% RNase at 37°C for 20 min and stained with 10 μ g/mL propidium iodide (PI, Sigma) for 1 hr at 4°C. 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 pol β gene in targeting cell

The existence, mRNA and protein expression of pol β gene 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 pol β in the targeting cell (Figure 1). RT-PCR and Western-blot both revealed that there was no expression of pol β in the pol β knockout cells (Figure 2, Figure 3).

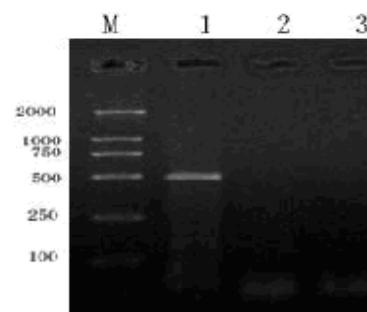


Figure 1. Agarose Gel Electrophoresis of PCR Products

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 pol β in the targeting cells. M: DNA Marker;

lane1: Control cells (EC9706); lane 2 and 3: polβ knockout EC9706 cells

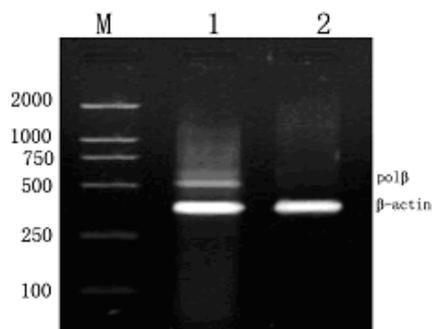


Figure 2. Agarose Gel Electrophoresis of RT-PCR Products

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

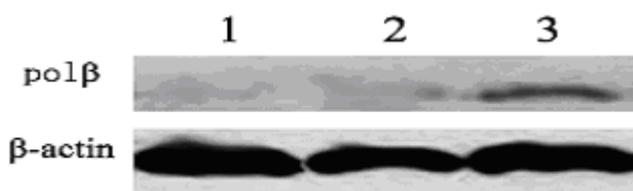


Figure 3. Western Blot Result

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.

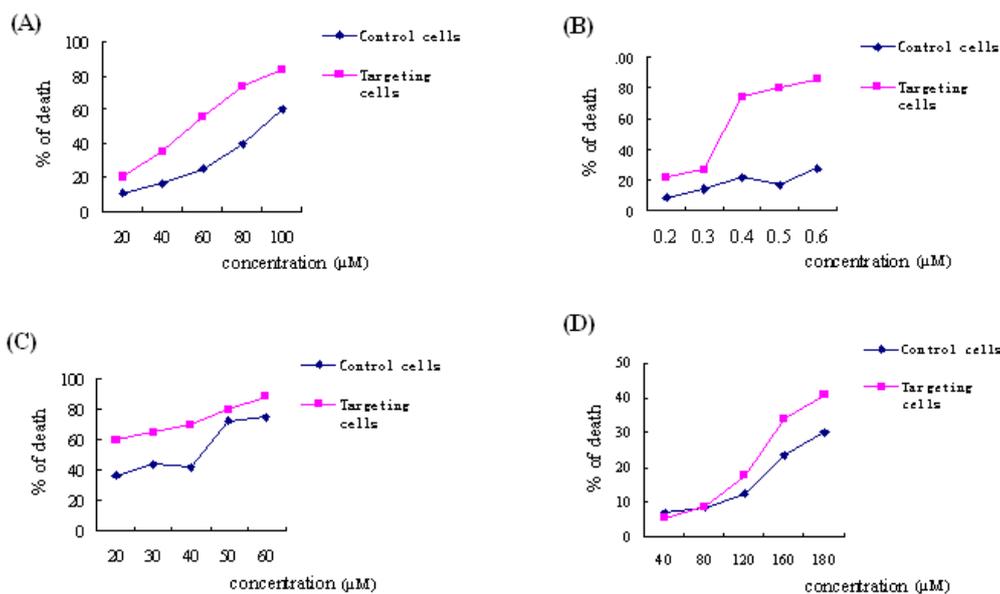


Fig.4 Cell sensitivity to DNA-damaging agents

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.

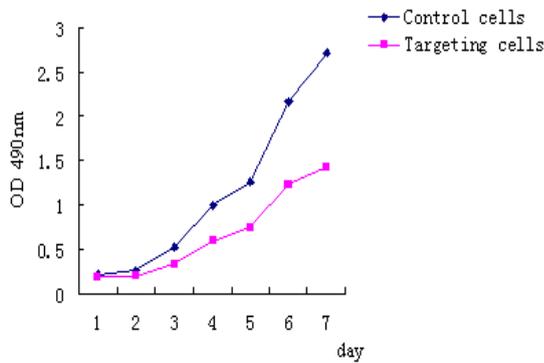


Fig.5 Proliferation curve of targeting cells and control cells

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
H ₂ O ₂	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 (%,x±s)

Group	G ₀ ~G ₁ (%)	S (%)	G ₂ ~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 pol β gene, finally, destroy the biological function of DNA pol β . The whole sequence was about 7.8kb (the upstream is 1280bp and the downstream is 1756bp). If occurred, pol β 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 pol β 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 pol β gene targeting cells were affected, mainly displayed depressant effect. However, overexpression of pol β gene may lead to continuous malignant transformation and accelerated growth velocity (23). Therefore, pol β 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 pol β 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 pol β while responding to oxidative stress has been shown in cultured cells and in organisms (25-26). In our research, pol β gene targeting cells were more sensitive to cisplatin, bleomycin and methylene blue, compared with control cell (EC9706). According to reference reports (27), pol β 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 pol β targeting cells while bleomycin was absolutely different. That may due to different expression level of pol β (pol β was deleted completely in targeting cells while there is very low level expression of pol β in knockdown cells).

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

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References

1. Hu DL, Zhuang ZX, Liu YM, He Y. Vector-mediated RNA interference of DNA polymerase beta in human bronchial epithelial cells. *Wei Sheng Yan Jiu*. 2006 Mar; 35(2):143-145.
2. Trivedi RN, Almeida KH, Fornasaglio JL, Schamus S, Sobol RW. The role of base excision repair in the sensitivity and resistance to temozolomide-mediated cell death. *Cancer Res*, 2005, 65 (14): 6394-6400.
3. Bu D, Cler LR, Lewis CM, Euhus DM. A variant of DNA polymerase beta is not cancer specific. *J Invest Surg*, 2004, 17(6): 327-331.
4. Miyamoto H, Miyagi Y, Ishikawa T, Ichikawa Y, Hosaka M, Kubota Y. DNA polymerase beta gene mutation in human breast cancer. *Int J Cancer*, 1999, 83(5): 708-709.
5. Iwanaga A, Ouchida M, Miyazaki K, Hori K, Mukai T. Functional mutation of DNA polymerase beta found in human gastric cancer--inability of the base excision repair in vitro. *Mutation Res*, 1999, 435(2):121-128.
6. Maria V. Sukhanova, Svetlana N. Khodyreva, Natalia A. Lebedeva, Rajendra Prasad, Samuel H. Wilson and Olga I. Lavrik. Human base excision repair enzymes apurinic/apyrimidinic endonuclease I (APE1), DNA polymerase beta and poly (ADP-ribose) polymerase 1: interplay between strand-displacement DNA synthesis and proofreading exonuclease activity. *Nucleic Acids Res*, 2005, 33 (4) :1222-1229.
7. Haitham T. Idriss, Osama Al-Assar, Samuel H, Wilson. DNA polymerase beta. *Int J Biochem Cell Biol*, 2002, 34:321-324.
8. Canitrot Y, Cazaux C, Fréchet M, Bouayadi K, Lesca C, Salles B, Hoffmann JS. Overexpression of DNA polymerase beta in cell results in a mutator phenotype and a decreased sensitivity to anticancer drugs. *Proc Natl Acad Sci USA*, 1998, 95:12586-12590.

9. Xu XC, el-Naggar AK, Lotan R. Differential expression of galectin-1 and galectin-3 in thyroid tumors. Potential diagnostic implications. *Am J Pathol.* 1995,147 (3): 815-822.
10. Kenneth C. Suen. Fine-needle aspiration biopsy of the thyroid. *CMAJ.* 2002, 167(5):491-495.
11. Akinari Iwanaga, Mamoru Ouchida, Kohji Miyazaki, Katsuji Hori and Tsunehiro Mukai. Functional mutation of DNA polymerase β found in human gastric cancer—inability of the base excision repair in vitro. *Mutat Res,* 1999, 435(2): 121-128.
12. Marian E. Eydmann and Margaret A. Knowles. Mutation analysis of 8p gene POLB and PPP2CB in bladder cancer · *Cancer Res,* 1997, 93(2):167-171.
13. Bergerheim US, Kunimi K, Collins VP, Ekman P. Deletion mapping of chromosomes 8, 10, and 16 in human prostatic carcinoma. *Gene Chromosom Cancer,* 1991, 3:215.
14. Dobashi Y, Shuin T, Tsuruga H, Uemura H, Torigoe S, Kubota Y. DNA polymerase beta gene mutation in human prostate cancer. *Cancer Res,* 1994, 54(9): 2827.
15. Ulrich Hübscher, Heinz-Peter Nasheuer and Juhani E. Syväoja. Eukaryotic DNA polymerases, a growing family. *TIBS,*2000,25(3):143-147 ·
16. Ziming Dong. Study of DNA polymerase β . *Journal of Zhengzhou University (Medical sciences),* 2003, 38(4): 477- 480.
17. Manu Kohli, Carlo Rago, Christoph Lengauer, Kenneth W. Kinzler and Bert Vogelstein. Facile methods for generating human somatic cell gene knockouts using recombinant adeno-associated viruses. *Nucleic Acids Res,* 2004, 32(1): e3.
18. Bunz,F. Human cell knockouts. *Curr. Opin. Oncol,* 2002,14: 73-78.
19. John M. Sedivy and Annie Dutriaux. Gene targeting and somatic cell genetics: a rebirth or a coming of age? *Trends Genet,* 1999, 15: 88-90.
20. Han Y, Wei F, Xu X, Cai Y, Chen B, Wang J, Xia S, Hu H, Huang X, Han Y, Wu M, Wang M. Establishment and comparative genomic hybridization analysis of human esophageal carcinomas cell line EC97O6. *Zhonghua YiXue Yi Chuan Xue Za Zhi.* 2002, 19(6): 455-457.
21. Xiujie Sheng. Strategy and development of gene targeting. *Journal of foreign medicine,* 2001, 24(1):8-10.
22. M Kozak. Effects of intercistronic length on the efficiency of reinitiation by eucaryotic ribosomes. *Mol Cell Biol,* 1987, 7:3438-3445.
23. Yamada NA, Farber RA. Induction of a low level of microsatellite instability by overexpression of DNA polymerase beta. *Cancer Res,* 2002, 62:6061-6064.
24. Lindahl T, Barnes DE. Repair of endogenous DNA damage. *Cold Spring Harb Symp Quant Biol.* 2000; 65:127-133.
25. Cabelof DC, Raffoul JJ, Yanamadala S, Guo Z, Heydari AR. Induction of DNA polymerase beta-dependent base excision repair in response to oxidative stress in vivo. *Carcinogenesis.* 2002 Sep; 23(9):1419-1425.
26. Chen KH, Yakes FM, Srivastava DK, Singhal RK, Sobol RW, Horton JK, Van Houten B, Wilson SH. Up-regulation of base excision repair correlates with enhanced protection against a DNA damaging agent in mouse cell lines. *Nucleic Acids Res.* 1998 Apr 15; 26(8):2001-2007.
27. Polosina YY, Rosenquist TA, Grollman AP, Miller H. 'Knock down' of DNA polymerase beta by RNA interference: recapitulation of null phenotype. *DNA Repair (Amst).* 2004 Nov 2; 3(11):1469-1474.