DCC Transfection alters gene expression and tumorigenic growth properties in Human MCF-7 Cell Line

Baoping Zhai*, Wentao Li, Linjiao Jia, Chao Ding, Gang Wu, Hui Zhi, Bing Zhang, Yuhong Li

Department of Breast Surgery, The People's Hospital of Zhengzhou University, The People's Hospital of Henan Province, Zhengzhou 450003, China
E-mail: zhaibaoping@163.com.

Abstract: Deleted in colorectal carcinoma (DCC) is an anti-oncogene which plays an important role in cell apoptosis and can induce cell cycle stasis, regulate cell adherence and migration. Many studies indicate that the abnormal expression of DCC can be found in many human tumors. Our experiment is to construct eukaryotic expressive vector and express the exogenous DCC gene and protein in the transfected cells MCF-7. We find that DCC gene plays an inhibitive role in tumorigenic growth properties in human MCF-7 cell line by flow cytometry detection of the transfected cells and growth curve analysis. Our experiment showed that the cell apoptosis rate of transfected cells, MCF-7/DCC increased obviously and DCC could inhibit the growth of MCF-7 . These results suggest that DCC gene may play a role in the breast cancer and development.
[Baoping Zhai, Wentao Li, Linjiao Jia, Chao Ding, Gang Wu, Hui Zhi, Bing Zhang, Yuhong Li. **DCC Transfection alters gene expression and tumorigenic growth properties in Human MCF-7 Cell Line.** *Life Sci J* 2013;10(2):1678-1681]. (ISSN:1097-8135). http://www.lifesciencesite.com. 235

Keywords: Breast carcinomas; Oncogene; DCC; Proliferation; Eukaryotic expressive vector; Gene transfection

Introduction

Deleted in colorectal carcinoma (DCC) is a tumor suppressor gene on chromosome 18q21.3, which includes 29 extrons, encoding RNA sequence about 4341bp in length and subsequent translating a transmembrane protein of approximate 1447 amino acids[1-3]. The DCC protein belongs to immunoglobulin superfamily[4]. Some studies have shown that DCC is involved in cell-cell adhesion. The abnormality of DCC gene can be found in many human tumors. Loss of DCC expression was observed in 20~60% of tumors from colorectal, nasopharyngus, gaster and lung[5-11]. DCC plays an important role on cell apoptosis and can induce cell cycle stasis, regulate cell adherence and migration in cells. DCC gene is a suppressor gene which has close relationship with tumorigenesis. The clinical pathological implications of DCC abnormalities in breast cancer have been investigated in a limited number of surgically resected breast cancer[5]. To further explore the role of DCC in the development and progression of breast cancer, we transfected exogenous DCC gene into MCF-7 cells.

Our pre-experiments show low expression DCC gene with different degree of the breast carcinomas[6]. It indicates that DCC gene maybe plays a role in the breast cancer and development. This study aims to transfect exogenous DCC gene into human breast cancer cell line MCF-7 in order to make normal DCC protein expressed by MCF-7 efficiently. It will provide the experiment basis for studying the relationship of DCC and MCF-7 tumorigenic growth properties, DCC and breast cancer cell cycle development.

Materials and methods

1 Materials:

1.1 Cell line and tissues: The human breast cancer cell line MCF-7 was preserved by our lab, cultivated with RPMI-1640 containing 10% fetal bovine serum under the enviroment of 37℃, 5%CO2. The colon tissue was taken from trauma patients underwent bowel resection surgery.

1.2 Plasmid and strains: The pIRES2-AcGFP1 plasmid was bought from Clontech company (American). E.coli JM109 strain was preserved by our lab.

1.3 Reagents: RPMI1640 medium and fetal bovine serum(GIBCO); LipofectamineTM2000 and Trizol(Invitrogen); Restriction enzymes such as *Xho* Ⅰ, *Sal* Ⅰ and One Step RT-PCR Kit (AMV), Plasmid Purification Kit, pMD18-T vector, T4DNA ligase (TaRaKa); Marker Ⅲ, Ⅳ, Ⅶ(Tianwei Times Company).

2 Methods

2.1 Construction of eukaryotic expressive vector: The tissue RNA was obtained with the technique of RT-PCR by using colon tissue samples preserved in liquid nitrogen and the primer with *Xho* Ⅰ and *Sal* Ⅰ enzyme cutting sites was used to amplify DCC gene. Then the PCR product was constructed into pMD-DCC recombinant plasmid. The positive clones was selected and identified by restrictive enzyme *Xho* Ⅰ and Sal Ⅰ and sequenced. Recombinant plasmid and pIRES2-AcGFP1 plasmid were digested by *Xho* Ⅰ and *Sal* Ⅰ restriction enzyme respectively, and then the reclaimed fragments DCC and pIRES2-AcGFP1 were joined by T4 ligase. The ligated products were transformed into competent cell JM109. The positive clones were screened by

ampicillin-resistance and clonal expansion. The plasmid was mini-extracted to identify with *Xho* Ⅰ and *Sal* Ⅰ restriction enzyme and sequencing.

2.2 Gene transfection and stable expressed cell lines screening: The MCF-7 cell line was cultured to log phase growth and collected to subcultivate into the six orifice plate for 24 hours. The transfection experiment group was added into transfection solution which was composited with pIRES2-AcGFP1/DCC plasmid and Lipofectamine, the control group was added into transfection solution which was composited with pIRES2-AcGFP1 empty plasmid and Lipofectamine. The transfected cell lines were cultured for 24 hours and then terminated with normal blood serum culture medium. When transfected cell lines were grew up to 80%, the cell lines were divided into different culture dishes according to 1:5 proportion and added into different concentration G418 to culture two weeks in order to screen stable expressed cell lines. The screened positive clone cell line was shifted into flask to culture continuously.

2.3 Detection the expression of exogenous DCC gene and protein in the transfected cells:

2.3.1 Expression of exogenous DCC gene was detected by RT-PCR: Using One-step RT-PCR kit(TaRaKa) to complete the reaction. The reaction system was 50 μl and the reaction condition was 50 ℃,30 min to run reverse transcription reaction; and then run the PCR reaction: 94 ℃,2 min;94 ℃,30 sec;55 ℃,30 sec;72 ℃,6 min;total 30 cycles. PCR products (6μl) were taken for detection by agarose electrophoresis.

2.3.2 Expression of DCC protein was detected by Western Blot: The total protein of transfected cell which had grown up to log phase was extracted and its concentration was to detect the DCC protein expression. 10% SDS-polyacrylamide separation gel and 4% SDS-polyacrylamide spacer gel were castinged respectively. The DCC protein sample was added into 25μl/hole. After electrophoresis the protein was transfered to NC membrane with semi-dry electro-transfer printing apparatus. Using defatted milk powder to close membrane and adding into TBST containing goat-anti-human DCC polyclonal antibody to react overnight under 4℃. The next day the membrane was washed three times and reacted with the secondary antibody (rabbit polyclonal antibody IgG) for 1 hour. The membrane was washed and reacted with ECL reagent, X-ray films were exposed, visualized and fixed in dark room. The negative control was phosphate buffer saline(PBS) instead of first antibody and positive control was Actin.

2.3.3 Expression of DCC protein was detected by Immunohistochemistry: Selecting the non-transfected and transfected MCF-7 cells which had grown up to log phase and digested by trypsinase to make monoplast cell suspension. The cells were subcultured into cultivation dish which was set cover glass beforehand. After 1 day cultivation, the cover glass was taken out, washed with PBS for 3 times, fixed by 4% paraformaldehyde and closed nonspecific antibody. The dealed cover glasses were added into first antibody(1:500 concentration of rabbit-anti-human DCC polyclonal antibody) to react 2 hours under 37℃, then were washed 3 times by PBS and added into secondary antibody(1:200 concentration of horseradish peroxidase labeled goat-anti-rabbit IgG) to react 2 hours under 37℃, washed 3 times by PBS. The cover glasses were mounted to observe and photograph under the light microscope.

2.3.4 Expression of DCC protein was detected by immunofluorescence staining: Selecting the non-transfected and transfected MCF-7 cells which had grown up to log phase and digested by trypsinase to make monoplast cell suspension. The cells were subcultured into cultivation dish which was set cover glass beforehand. After 1 day cultivation the cover glass was taken out, washed with PBS for 3 times, fixed by 4% paraformaldehyde and closed nonspecific antibody. The dealed cover glasses were added into first antibody(1:500 concentration of rabbit-anti-human DCC polyclonal antibody) to react 2 hours under 37℃, then were washed 3 times by PBS and added into secondary antibody(1:200 concentration of FITC labeled goat-anti-rabbit IgG) to react 2 hours under 37℃, washed 3 times by PBS. The cover glasses were mounted to observe and photograph under the fluorescence microscope.

2.4 Flow cytometry detection of transfected cells: Collecting the MCF-7 cell lines which could express stably pIRES2-AcGFP1/DCC and had been identified by PCR,Western Blot and Immunohistochemistry to synchronize and culture the cells for 48 hours. The synchronized cells were digested by trypsinase and fixed by 70% ethanol for 24 hours under 4℃. The samples' RNA was digested by RNase and stained with PI solution. So the samples could be measured on the flow cytometry. The distribution of cell cycle was analyzed with Multicycle for Windows software and apoptosis rate was calculated with CellQuest software.

2.5 Growth curve analysis: Non-transfected and transfected MCF-7 cells were digested by 0.25% trypsinase to make monoplast cell suspension, then were subcultured into six orifice plate. Every kind of cell was subcultured six holes, 1×10^4 cells/hole. Every day taking one hole cells to count and finally to draw the cell growth curve.

2.6 Proliferation activity of non-transfected and transfected cells by MTT: The transfected DCC clonal cells(MCF-7/DCC), transfected empty vector pIRES2-AcGFP1 cells(MCF-7/vect) and

non-transfected MCF-7 cells were subcultured into 96 orifice plate according to 1×10^4/hole propotion respectively. The cells were cultured for 0 hour, 24 hours, 48 hours, 72 hours; removed medium, washed by PBS for 2 times, added into 20 μl MTT solution(5g/L) /hole and reacted under 37 ℃ for 4 hours. The staining solution was removed, and added into 150 μl DMSO/hole, shaked on the level swing bed and read the A value under 490 nm of the enzyme-mark apparatus.

3 Statistical treatment:

All data were indicated with mean±standard deviation (mean±SD). SPSS13.0 analysis software was used to carry out AVONA analysis. $P<0.05$ indicated that the data variance had statistical significance.

Results

1 Identification of pIRES2-AcGFP1/DCC with enzyme-cutting and sequencing: The constructed recombinant plasmid pIRES2-AcGFP1/DCC could be cut into two fragments: 4341bp DCC target gene and 5300bp pIRES2-AcGFP1(Fig1). Sequencing result indicated that its sequence coincided with the DCC on the GenBank.

2 Identification of DCC expression in the MCF-7 cell: The screened positive clones MCF-7 cells were taken RT-PCR reaction and could amplify DCC total gene length, while the pIRES2-AcGFP1 transfected and non-transfected MCF-7 cells were taken the same reaction and a little DCC gene fragment appeared. The transfected MCF-7/DCC cells showed 185 KD strap with Western Blot. Immunohistochemical staining indicated that the transfected MCF-7/DCC cells' membrane and cytoplasm had evident brown particles or patching stain, while MCF-7/vect cells and non-transfected MCF-7 cells only had few positive particles in the cell membrane and cytoplasm. This result showed that exogenous DCC protein could be expressed in the MCF-7/DCC cells. Immunofluorescence staining indicated that the transfected MCF-7/DCC cells' membrane and cytoplasm had evident fluorescent particles, while MCF-7-vect cells and non-transfected MCF-7 cells only had few positive particles in the cell membrane and cytoplasm. This result showed that the MCF-7/DCC cells could express exogenous DCC protein.

3 Cell cycle and apoptosis analysis of transfected MCF-7 cells: Cell cycle analysis revealed that the transfected MCF-7/DCC cells cell cycle S phase extended obviously. Comparing with the transfected MCF-7/vect cells and non-transfected MCF-7 cells, the transfected MCF-7/DCC cells apoptosis rate increased obviously.

4 Detection of MCF-7 cell proliferation activity in vitro with MTT: The three tumor cells MCF-7/DCC,MCF-7/vect and MCF-7 cells had no evident difference in the grow morphous under microscope. The MCF-7 cells' proliferation activity in vitro was detected by MTT method. There was statistical significance in the value when comparing MCF-7/DCC cells with the two control groups ($P<0.05$). While the value had no statistical significance between MCF-7/vect and MCF-7 cells ($P>0.05$). These results indicated that DCC protein could inhibit the proliferation of MCF-7 cells.

5 Analysis of transfected MCF-7 cells growth condition: The data showed that the transfected MCF-7/DCC cells' growth velocity was slower than the parental MCF-7 and MCF-7/vect cells.

Discussion

DCC is an anti-oncogene which has close relation with tumorigenesis. The abnormal expression of DCC can be found in many human tumors. Many studies have shown that DCC gene has low expression to different degrees in the colorectal carcinoma, nasopharyngeal carcinoma, lung cancer and gaster cancer[12-14]. DCC not only can induce cell cycle stasis and play an important role in cell apoptosis, but also can regulate cell adherence and migration[15-16].

Our pre-experiments have shown that the expression of DCC gene was low in the breast cancer, while it was high in the peri-carcinoma tissues. It indicates that DCC gene may play an important role in the carcinogenesis and development of breast cancer. The studies indicate that repairing and adding antioncogene which was inactivated or deleted from carcinoma cells through gene transfection method can inhibit the carcinoma cells growth, which mainly depends on the high and stable expression of exogenous antioncogene. To study the effect of DCC gene on the breast carcinoma biological behaviour, we should first make the exogenous DCC gene expressed stably and highly in the breast carcinoma cells which deleted the DCC gene, that is we need the cell line that DCC protein can be expressed stably and highly.

The experiment analyzes the effect of DCC gene on the human breast carcinoma cell MCF-7 on the basis of DCC gene cloning. We use the human DCC gene to transfect MCF-7 human breast carcinoma cell line. With clone screening we get the DCC high expression cell line (MCF-7/DCC). With PCR, Western Blot and Flow cytometry analysis the results show that DCC gene can be stably expressed in the transfected MCF-7 cells, DCC gene can change cell cycle of carcinoma cell, make the MCF-7 cell S phase extended obviously, inhibit MCF-7 cell growth and induce the breast cancer cell apoptosis. These data provide the evidence for further study on DCC gene therapy.

Corresponding author:
Baoping Zhai, Department of Breast Surgery, The People's Hospital of Zhengzhou University, The People's Hospital of Henan Province, Zhengzhou 450003, China. E-mail: zhaibaoping@163.com.

References

[1] Duman-Scheel M. Deleted in Colorectal Cancer (DCC) pathfinding: axon guidance gene finally turned tumor suppressor.Curr Drug Targets, 3(11):1445-1453.
[2] Norris AD, Lundquist EA. UNC-6/netrin and its receptors UNC-5 and UNC-40/DCC modulate growth cone protrusion in vivo in C. elegans.Development,138(20):4433-4442.
[3] Lassus H, Salovaara R, Aaltonen LA, Butzow R.Allelic analysis of serous ovarian carcinoma reveals two putative tumor suppressor loci at 18q22-q23 distal to SMAD4, SMAD2, and DCC.Am J Pathol,159(1):35-42.
[4] Hao JC, Adler CE, Mebane L, Gertler FB, Bargmann CI, Tessier-Lavigne M. The tripartite motif protein MADD-2 functions with the receptor UNC-40 (DCC) in Netrin-mediated axon attraction and branching.Dev Cell, 18(6):950-60.
[5] Srour M, Rivière JB, Pham JM, Dubé MP, Girard S, Morin S, Dion PA, Asselin G, Rochefort D, Hince P, Diab S, Sharafaddinzadeh N, Chouinard S, Théoret H, Charron F, Rouleau GA.Mutations in DCC cause congenital mirror movements .Science, 328(5978):592.
[6] Zhai Bao-ping, Zhang Bing, Li Wen-tao. Clinical Significance for Expression of DCC in Human Breast Carcinomas. Chinese Journal of Experimental Surgery. 27(11) 1669-1670.
[7] Yang L, Garbe DS, Bashaw GJ.A frazzled/DCC-dependent transcriptional switch regulates midline axon guidance.Science. 2009 May 15;324(5929):944-7.
[8] Mattar R, Nonogaki S, Silva C, Alves V, Gama-Rodrigues JJ.P53 and Rb tumor suppressor gene alterations in gastric cancer.Rev Hosp Clin Fac Med Sao Paulo,59(4):172-180.
[9] Ramburan A, Chetty R, Hadley GP, Naidoo R, Govender D.Microsatellite analysis of the DCC gene in nephroblastomas: pathologic correlations and prognostic implications.Mod Pathol,17(1):89-95.
[10] Inokuchi K, Yamaguchi H, Hanawa H, Tanosaki S, Nakamura K, Tarusawa M, Miyake K, Shimada T, Dan K.Loss of DCC gene expression is of prognostic importance in acute myelogenous leukemia. Clin Cancer Res, 8(6):1882-1888.
[11] Eide CA, Adrian LT, Tyner JW, Mac Partlin M, Anderson DJ, Wise SC, Smith BD, Petillo PA, Flynn DL, Deininger MW, O'Hare T, Druker BJ.The ABL switch control inhibitor DCC-2036 is active against the chronic myeloid leukemia mutant BCR-ABLT315I and exhibits a narrow resistance profile.Cancer Res,71(9):3189-3195.
[12] Derks S, Bosch LJ, Niessen HE, Moerkerk PT, van den Bosch SM, Carvalho B, Mongera S, Voncken JW, Meijer GA, de Bruïne AP, Herman JG, van Engeland M. Promoter CpG island hypermethylation- and H3K9me3 and H3K27me3-mediated epigenetic silencing targets the deleted in colon cancer (DCC) gene in colorectal carcinogenesis without affecting neighboring genes on chromosomal region 18q21.Carcinogenesis,30(6):1041-1048.
[13] Furne C, Corset V, Hérincs Z, Cahuzac N, Hueber AO, Mehlen P. The dependence receptor DCC requires lipid raft localization for cell death signaling.Proc Natl Acad Sci U S A,103(11):4128-4133.
[14] Manal A. Shalaby, Howaida A. Nounou, Maha Arafah. Identification of Plexin D1as a tumor biomarker in brain, breast and thyroid cancers.Life Science Journal,10(1)2647-2652.

5/27/2013