RESEARCH ARTICLE

Construction and Expression of an Eukaryotic Expression Vector Containing the IER3 Gene

Zhen Wang[1], Hong-Sheng Yu[1], Ru-Yong Yao[2], Wen-Sheng Qiu[1], Lu Yue[1], Ai-Hua Sui[2], Xiang-Ping Liu[2], Shi-Hai Liu[2]*

Abstract

Background: More and more research indicate that the immediately early response gene 3 (IER3) is involved inmany biological provesses, such as apoptosis and immunoreaction, as well as viral infection, tumorigenesis and tumour progression. Methods: Here we describe the construction of an eukaryotic expression vector containing IER3 gene and its expression in A549 cells as assessed through fluorescence microscopyand Western-blotting. Results: Fluorescence detection displayed that GFP in cytoplasm was high during 48 and 72 hours post-transfection. In addition, Western blotting showed significant increase in IER3 gene expression in the transfected cells compared with controls. Conclusion: The recombinate plasmid expression vector was constructed successfully, which may provide a basis for further exploration of function of IER3 in lung cancer.

Keywords: IER3 gene - eukaryotic expression vector - tumor

Asian Pacific J Cancer Prev, **14 (1)**, 507-510

Introduction

IER3 gene, also known as IEX-1, p22/PRG1, Dif-2, murine96 and so on, is located on the short arm of the chromosome 6 (6p21.3) near to the MHC locus (Billmann-Born et al., 2011; Han et al., 2011; Santamaria et al., 2012). It consists of two exons (respectively 440 bp and 890 bp) and a 112 bp intron (Arlt et al., 2011). First discovered from cutaneous squamous cell carcinomous induced by UV radiation, IER3 gene interacts with variety of signaling pathways such as NF-κB/rel, P53, c-myc and so on (Yang et al., 2002; Luisi et al., 2011). Recent research indicates that IER3 gene plays a vital role in the regulation of physiology including apoptosis, immunoreaction, viral infection as well as tumorigenesis and tumor progression (Lee et al., 2006; Ao et al., 2008).

It is estimated that there were 1.61 million cases of lung cancer diagnosed worldwide in 2008, representing about 12.7% of all new cancers globally (Schwander et al., 2012). It is the most common cancer among men and the second most common among women (Askoxylakis et al., 2010). A549 cells is a valuable model in study pathogenesis and treatment of lung cancer (Wang et al., 2012). Gene therapy vectors are particularly promising, Plasmid-based vectors are capable of achieving a stable and efficient gene expression in target cells in vitro (Sipo et al., 2006).

In the present report, we were intended to construct an eukaryotic expression vector containing IER3 gene and subcloned into pcDNA3.1-6His vector whose recombinant could be expressed in A549 cells. After transfected correctly, the functional localization of target protein could be observed by fluorescence microscope, which establish the basis for the further exploration of IER3 gene.

Materials and Methods

Reagents and cell cultures

E.coli DH5α, A549 lung adenocarcinoma cell lines and double antibiotic solution are all preserved by the Central Laboratory of the Affiliated Hospital of Medical College QingDao University; pGEMT easy vector and pcDNA3.1-6His vector are both from Promega Corporation; TRIzol and SYBR Green are from Invitrogen Corporation. Reverse transcription kit, Ex taq Hot Start Version, T4 DNA ligase, Restriction endonuclease and DL2000 marker are all from TaKaRa Corporation. Gel Extraction Mini Kit and Plasmid Purification Mini Kit are from Watson Biotechnologies, Inc. Potassium acetate, Tryptone and Sodium chloride are all from Sigma USA.

A549 cells were grown in DMEM medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (Invitrogen, USA). Cells were maintained at 37 °C and 5% CO2 during all exprements.

Construction of an eukaryotic expression vector

According to IER3 nucleotide sequence from GeneBank, four pairs of detecting primers were designed

[1]Department of Oncology, [2]Central Laboratory, Affiliated Hospital of Medical College, Qingdao University, Qingdao, China
**For correspondence: shihailiu2012@163.com*

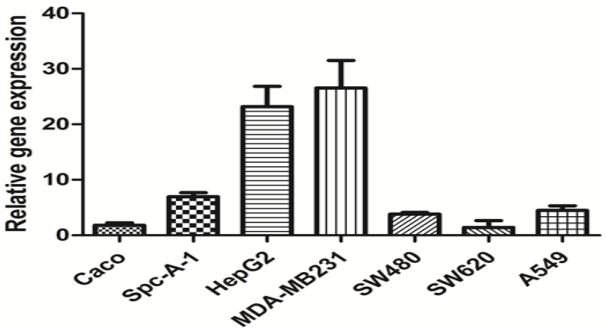

Figure 1. The Expression of IER3 in Different Tumor Cell Lines

to detect IER3 gene expression level from seven different cell lines including Caco, Spc-A-1, HepG2, MDA-MB231, SW480, SW620 and A549. In the end A549 cell lines was selected as our object of study because of its equivalent expression of IER3 gene. Gene-specific primers and reverse transcriptase were used to convert mature miRNA to cDNA The PCR products as well as DL2000 marker were submitted to 2% agarose gel and the target stripe was extracted and purified by Gel Extraction Mini Kit (Watson Shanghai).

The construct of the ligation of pGEMT-IER3 was transformed into competent cell DH5α. Selected by ampicillin, the positive clone was cultured in LB medium containing ampicillin. After 12 h, the recombinant plasmid was extracted and digested by EcoR I. Then, the extracted plasmid were sent to shanghai sangon to be confirmed by

DNA sequencing technology.

Digested by Xba I and BamH I, the cloned IER3 gene was subcloned into pcDNA3.1-6His vector containing the same restriction enzyme cutting site by T4 DNA ligase (TaKaRa Dalian) in the condition of 22.5 °C constant temperature for 2 h. To prove it successful, we took 5 μl construct to submitted to 2% agarose gel.

Gene transfection

When the lung adenocarcinoma epithelial cell line A549 cells density reached to 1×10^8/L, the cells were inoculated into six orifice plate and transfected with the recombinant pcDNA3.1-6His-IER3 and its control groups including transfecting only pcDNA3.1-6His easy vector as well as equal DMEM medium using liposome. The cells were transfected for 72 h, harvested and extracted total RNA and protein.

Detecting IER3 gene expression

48 h post-transfection of pcDNA3.1-6His-IER3, we observed the IER3 gene expression by inverted flurescence microscopy and its functional localization by immunofluorescent staining.

Nextly, we prove the vector sucessfuly transfected by western blot. Samples were heated (95 °C) for 5 min and submitted to SDS-PAGE using 4%–20% Tris –glycine gradient gels. After electroblotting onto polyvinylidene difluoride membrane (PVDF), blots were blocked for 1 h with 3% nonfat milk powder, 0.1% bovine serum albumin in TBS (Blotto) at room temperature. Blots were exposed to the primary antibodies diluted in Blotto

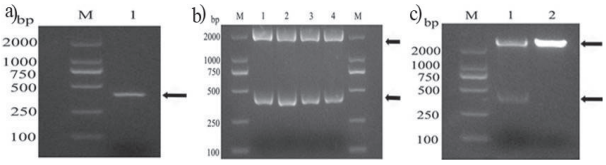

Figure 2. Identification of IER3 Gene by Electrophoresis. a) The amplification of IER3 gene. M: DL2000; Lane 1: The results of IER3 gene PCRThe arrowheads implies the purpose fragment 468 bp; b) The identification of pGEMT easy-IER3 vector. M: DL2000; Lane 1-12: pGEMT-IER3 vector cleaved by EcoR I, in which 1, 2, 3, 4 is positive clone; The arrowheads respectively implies the vector and purpose fragment; c) The identification of pcDNA3.1-6His-IER3 vector. M: DL2000; Lane 1-3: pcDNA3.1-6His-IER3 cleaved by Xba I and BamH I. The arrowheads respectively implies the vector and purpose fragment

overnight at 4 °C. After three washes with Blotto, blots were exposed to the appropriate horseradish peroxidase-conjugated secondary antibody diluted in Blotto for 1 h. Blots were then washed three times in TBS, developed with Super-Signal West Dura solution, and analyzed with the Chemidoc system.

Statistical analysis

Data was all expressed as mean ± standard (SD). Statistical significance was computed by two-tailed unpaired Studeng's test in which p values less than 0.05 was considered as statistically significant in SPSS14.0.

Results

Choose the appropriate cell lines

By means of the best selected primers seven different cell lines were all identified by qPCR to detect their IER3 expression level. Considering our futural research on lung cancer, we choose the A549 cell which expressing little IER3 protein as our target transfected cells (Figure 1) .

Amplification of and identification of pGEMT-IER3

Gene-specific primers and reverse transcriptase were used to convert mature miRNA to cDNA, Through PCR technology, these cDNA were turned into dDNA under the roles of SYBR. The result of the 2% agarose gel electrophoresis showed that IER3 gene was amplified correctly (Figure 2a) .

The successful conduct of ligation transformed into DH5α cells was amplified by E.coli DH5α. By plasmid purification and restriction enzyme digestion, the monoclonal bacterial colony was proved to be right. (Figure 2b) .

Construction of pcDNA3.1-6His-IER3 and its transfection

Digested by Xba I and BamH I, the pcDNA3.1-6His-IER3 vector was divided into two pieces including the target fragment and the expression vector fragment. (Figure 2c) .

The conduct encoding IER3 protein was transfected into A549 cells through liposome. 48 hours after transfection, cells harboring a red expressing integrant were viewed by inverted flurescence microscopy. After 72 hours monitoring, the result suggested that the GFP expression was high post transfection. The

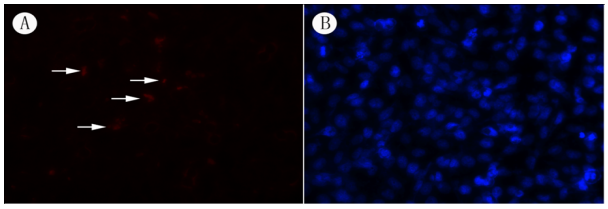

Figure 3. A549 Cells Transfected with pcDNA3.1-6His-IER3. A: The tintage of pcDNA3.1-6His-IER3; B: The result of DAPI tintage. The arrowheads implies positive cells transfected correctly

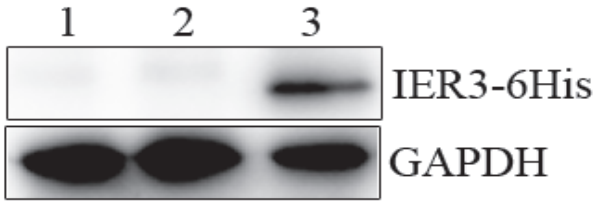

Figure 4. Detection of IER3 Expression by Western Blot. 1) A549 cells; 2) A549 cells were transfected with pcDNA3.1-6His; 3) A549 cells were transfected with pcDNA3.1-6His-IER3

immunofluorescent staining showed that IER3 protein mainly located in cytoplasm (Figure 3) .

Western blotting

Three groups of cells were all identified by western blot. Compared with another two groups (blank control, pcDNA3.1-6His transfection only), the experimental group (pcDNA3.1-6His-IER3 transfection) expressed IER3 protein higher, which suggested that the expression vector was expressed correctly in A549 cells whereas the other two groups influence little on IER3 gene (Figure 4).

Discussion

IER3, a kind of apoptotic regulatory gene, plays an important role in homeostasis and various of disease especially in tumorigenesis and caner progression (Segditsas et al., 2008). A lot of stimulation such as ionizing radiation, growth factors (TNF-α, IL-1β), virus infection, tumor-promoting phorbol ester, lipopolysaccharide (LPS) and so on could irritate rapid and transient IER3 expression in cells with the need of salvage synthesis pathway which reaches the peak within 15-20 min (Zilliox et al., 2007). It participate in cellular stress, proliferation and apoptosis mainly through NF-ϰB/rel (Huang et al., 2002; Wu, 2003), p53 (Im et al., 2002), c-myc (Wu, 2003) and other tumor-ralated gene.

The prior researches showed that hyper methylation in the region of its promoter was ralated to malignant hemopathy (Krieger et al., 2005). Morever, IER3 gene is involved with breast cancer (Hu et al., 2004), pancreatic cancer (Sasada et al., 2008), ovarian cancer (Lee et al., 2012) and so on. The pleiotropic effects of IEX-1 on cell death and differentiation critically depend on the cellular context, yet the concrete mechanism of IER3 gene is not very clear. There is three hypothesis that was gotten scholars' consensus. The fist one is that IER3 gene may combine FIFOATP complex in mitochondria, which regulates the level of the ATP and active oxygen free radicals (ROS) whose role is very important in cancer cells' apoptosis (Akilov et al., 2012). Another mechanism is involved in cellular immunity. Cytotoxic T cells couldn't recognize tumor cells' limitness proliferation because they are lack of surface antigen. IER3 could encode an antigenic epitope which could be recognized by Cytotoxic T cells (Matsueda et al., 2007).

In addition, there are at least four isoforms of IEX-1 with molecular weight of 18, 22, 29, or 31 kDa, respectively owing to post-translation modifications or alternative splicing (Steensma et al., 2009). The ratio of these isoforms or their differential distributions among cytoplasm, nuclei, and intracellular membranes may also play a role in determination of the outcome of IER3. Unfortunately, there are no available Abs or other means yet to distinguish these forms and to test their functions individually.

This research successfully constructed an eukaryotic expression vector containing IER3 gene which was transfected into A549 cells to observe its expression by inverted flurescence microscopy as well as its functional localization by immunofluorescent staining, which provides a basis for futural research on IER3 gene. Such a study is particularly important at present, radiation and many chemotherapy drugs induce IER3 expression at relatively high levels that may contribute to treatment to tumorigenesis, which may lead to increased efficacies in therapeutic intervention of lung cancer.

Acknowledgements

During the research, authors acknowledged the valuable help rendered by professor Ye Liang and Kun Yang, Central Laboratory, Affiliated Hospital of Medical College, Qingdao University. Besides, this study is supported by Shandong Natural Science Foundation (Y2008C48); Shandong Excellent Young Scientist Research Award Fund Project (BS2010YY013). Shandong Tackle Key Problems in Science and Technology (2010GSF10245). Youth Fundation of The Affiliated Hospital of Medical College, Qingdao University (AHMCQ201232); Department of Education of Shandong Province (J11LF65).

References

Akilov OE, Wu MX, Ustyugova IV, et al (2012). Resistance of Sezary cells to TNF-alpha-induced apoptosis is mediated in part by a loss of TNFR1 and a high level of the IER3 expression. *Exp Dermatol*, **21**, 287-92.

Ao L, Liu JY, Gao LH, et al (2008). Differential expression of genes associated with cell proliferation and apoptosis induced by okadaic acid during the transformation process of BALB/c 3T3 cells. *Toxicology In Vitro*, **22**, 116-27.

Arlt A, Schafer H (2011). Role of the immediate early response 3 (IER3) gene in cellular stress response, inflammation and tumorigenesis. *Eur J Cell Biol*, **90**, 545-52.

Askoxylakis V, Thieke C, Pleger ST, et al (2010). Long-term survival of cancer patients compared to heart failure and stroke: a systematic review. *BMC Cancer*, **10**, 105.

Billmann-Born S, Till A, Arlt A, et al (2011). Genome-wide expression profiling identifies an impairment of negative

feedback signals in the Crohn's disease-associated NOD2 variant L1007fsinsC. *J Immunol*, **186**, 4027-38.
De Luisi A, Ferrucci A, Coluccia AM, et al (2011). Lenalidomide restrains motility and overangiogenic potential of bone marrow endothelial cells in patients with active multiple myeloma. *Clin Cancer Res*, **17**, 1935-46.
Han L, Geng L, Liu X, et al (2011). Clinical significance of IEX-1 expression in ovarian carcinoma. *Ultrastruct Pathol*, **35**, 260-6.
Huang YH, Wu JY, Zhang Y, et al (2002). Synergistic and opposing regulation of the stress-responsive gene IEX-1 by p53, c-Myc, and multiple NF-kappaB/rel complexes. *Oncogene*, **21**, 6819-28.
Hu Y, Sun H, Drake J, et al (2004). From mice to humans: identification of commonly deregulated genes in mammary cancer via comparative SAGE studies. *Cancer Res*, **64**, 7748-55.
Im HJ, Pittelkow MR, Kumar R (2002). Divergent regulation of the growth-promoting gene IEX-1 by the p53 tumor suppressor and Sp1. *J Biol Chem*, **277**, 14612-21.
Krieger S, Grunau C, Sabbah M, et al (2005). Cyclin D1 gene activation in human myeloma cells is independent of DNA hypomethylation or histone hyperacetylation. *Exp Hematol*, **33**, 652-59.
Lee S, Bang S, Song K, et al (2006). Differential expression in normal-adenoma-carcinoma sequence suggests complex molecular carcinogenesis in colon. *Oncol Rep*, **16**, 747-54.
Lee YH, Kim JH, Zhou H, et al (2012). Salivary transcriptomic biomarkers for detection of ovarian cancer: for serous papillary adenocarcinoma. *J Mol Med (Berl)*, **90**, 427-34.
Matsueda S, Takedatsu H, Sasada T, et al (2007). New peptide vaccine candidates for epithelial cancer patients with HLA-A3 supertype alleles. *J Immunother*, **30**, 274-81.
Santamaria C, Ramos F, Puig N, et al (2012). Simultaneous analysis of the expression of 14 genes with individual prognostic value in myelodysplastic syndrome patients at diagnosis: WT1 detection in peripheral blood adversely affects survival. *Ann Hematol*, **91**, 1887-95.
Sasada T, Azuma K, Hirai T, et al (2008). Prognostic significance of the immediate early response gene X-1 (IEX-1) expression in pancreatic cancer. *Ann Surg Oncol*, **15**, 609-17.
Schwander B, Ravera S, Giuliani G, et al (2012). Cost comparison of second-line treatment options for late stage non-small-cell lung cancer: cost analysis for Italy. *Clinicoecon Outcomes Res*, **4**, 237-43.
Segditsas S, Sieber O, Deheragoda M, et al (2008). Putative direct and indirect Wnt targets identified through consistent gene expression changes in APC-mutant intestinal adenomas from humans and mice. *Hum Mol Genet*, **17**, 3864-75.
Sipo I, Hurtado Pico A, Wang X, et al (2006). An improved Tet-On regulatable FasL-adenovirus vector system for lung cancer therapy. *J Mol Med (Berl)*, **84**, 215-25.
Steensma DP, Neiger JD, Porcher JC, et al (2009). Rearrangements and amplification of IER3 (IEX-1) represent a novel and recurrent molecular abnormality in myelodysplastic syndromes. *Cancer Res,* **69**, 7518-23.
Wang H, Wei F, Zhang J, et al (2012). A novel immunocompetent murine tumor model for the evaluation of RCAd-enhanced RDAd transduction efficacy. *Tumour Biol*, **33**, 1245-53.
Wu MX (2003). Roles of the stress-induced gene IEX-1 in regulation of cell death and oncogenesis. *Apoptosis*, **8**, 11-8.
Yang JJ, Preston GA, Alcorta DA, et al (2002). Expression profile of leukocyte genes activated by anti-neutrophil cytoplasmic autoantibodies (ANCA). *Kidney Int*, **62**, 1638-49.
Zilliox MJ, Moss WJ, Griffin DE (2007). Gene expression changes in peripheral blood mononuclear cells during measles virus infection. *Clin Vaccine Immunol*, **14**, 918-23.