

Electro-gene therapy followed by intratumoral injection of pcDNA3.1-p27^{Kip1} wild type in human tongue base cancer cells sp-c3 xenograft

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ABSTRACT

Human tongue base cancers are characterized by a high degree of local invasion and metastasis to the regional lymph nodes and included a disease with difficult treatment. A novel method for high-efficiency and region-controlled *in vivo* gene transfer was developed by combining electro-gene therapy and plasmid (pcDNA). The aims of the study were to examine the efficiency of transfection of p27^{Kip1} gene by electro-gene therapy, and to evaluate p27^{Kip1} gene therapy in *Supri's clone-3* (SP-C3) xenografts using pcDNA3.1-p27^{Kip1} wild type (wt) and pcDNA3.1 empty vector (neo) with electro-gene therapy. To investigate gene transfer method, the enhanced green fluorescence protein (EGFP) gene was transfected into xenografts by electro-gene therapy. The efficiency of p27^{Kip1} gene transfection at protein level was confirmed by Western blotting. To estimate the reduction of tumor size in Wistar Balb/c mice after electro-gene therapy with p27^{Kip1} wt gene was examined by tumorigenesis assay. To evaluate the induction of apoptosis was carried out by colorimetric assay. Result, the growth of tumors was markedly suppressed by p27^{Kip1} wt gene transfection. Up-regulation of p27^{Kip1} protein was detected in pcDNA3.1-p27^{Kip1} wt. Apoptosis induction through activity of caspase -3 and -9 were significantly increased in p27^{Kip1} wt-transfected tumors. These results suggest that it is possible to transfer p27^{Kip1} wt into tongue base cancer cell xenografts using electro-gene therapy. p27^{Kip1} wt had a high-potentially to suppress the growth of tumors. Conclusion, electro-gene therapy followed by intratumoral injection of pcDNA3.1-p27^{Kip1} wt had a high-potentially to suppress the growth of a human tongue base cancer cell xenograft.

Key words: pcDNA3.1-p27^{Kip1} wt, electro-gene therapy, tongue base cancer SP-C3, xenograft

INTRODUCTION

Electro-gene therapy or electroporation or electric pulse has been developed for the purpose of achieving highly efficient *in vitro* gene or drug transfer.^{1,2} This system provides markedly higher efficiency transfer compared with other non-viral transfer system, including cationic liposome.³

Electro-gene therapy has been applied to *in vivo* drug transfer for cancer treatment and clinical trial has been started.⁴ Electro-gene therapy has become more and more popular as an effective technique for introduction of foreign DNA into cells of various kinds of mammalian cells.^{5,6} for investigation of gene regulation⁷, and has been demonstrated to be highly useful in transfecting

human hematopoietic stem cells for gene therapy.⁸ However, the transfection efficiency in mammalian cells using in vivo electro-gene therapy has received little attention⁹ and usually is still low, typically about 0.01-1%.¹⁰ Because electro-gene therapy is a physical method, it has a little biological side effect and is free of chemical toxicity.⁷

Many types of methods and techniques for in vivo gene transfer have been developed, and some of them have already been applied in clinical trials.¹ Non-viral gene transfer, “naked” plasmid DNA is an ideal system for gene transfer. A plasmid mediated method would be economical and easy because use of this system obviates the necessity to construct viral vectors, establish clones of producer cells, access viral titers and presence of replication-competent helper virus, which has been known to activate passive oncogenes. The transfer procedure could be easily repeated because “naked” plasmid DNA has little antigenicity to the host body.¹¹

Human tongue base cancers are characterized by a high degree of local invasion and a high rate of metastases to the cervical lymph nodes. Moreover, human tongue base cancer frequently shows local recurrence after initial treatment, probably due to micro invasion and/or metastasis of tumor cells at the primary site.¹² SP-C3 cell line has an extremely growth, highly invasion and metastasis to the regional and distant lymph nodes, rapidly local recurrence after radical excision, highly mortality, and included a disease with difficult treatment.¹³ Despite advanced in surgery, radiotherapy and chemotherapy, the survival of patients with oral tongue cancer has not significantly improved over the past several decades. Also, treatment options for recurrent or refractory oral cancers are limited.¹⁴ Furthermore, the ratio of mortality or incidence in 1980 and 1990 was 0.48 and 0.47, respectively¹⁵ and the prognosis has not changed during the past 10 years. However, as a new strategy for refractory cancer, gene therapy is watched with keen interest.

p27^{Kip1} is a universal cyclin-dependent kinase inhibitor that directly inhibits the enzymatic activity of cyclin-CDK complexes, resulting in cell cycle arrest at G₁.¹⁶ p27^{Kip1} has an important prognostic factor in various malignancies. Recently, decreased expression of p27^{Kip1} has been frequently detected in human cancer.¹⁷⁻¹⁹ In

addition, loss of p27^{Kip1} has been associated with disease progression and an unfavorable outcome in several malignancies.²⁰ Furthermore, mice lacking the p27^{Kip1} gene show an increase in body weight, thymic hypertrophy and hyperplasia of pituitary intermediate lobe adrenocorticotrophic hormone cells, adrenal glands and gonadal organ.²¹ Moreover, malignant human oral cancer cells transfection with p27^{Kip1} gene leads to inhibition of proliferation, invasion and metastasis.^{22,23}

In the present study, the efficiency of transfection of exogenous p27^{Kip1} gene by electro-gene therapy, and the antitumor activity of p27^{Kip1} gene therapy in human tongue base cancer cells *Supri's clone-3* (SP-C3) xenografts using pcDNA3.1-p27^{Kip1} wild type (wt) and pcDNA3.1 empty vector (neo) with the local application of electric pulses was evaluated.

MATERIALS AND METHODS

Cell and cell culture. SP-C3 cells were isolated from a cervical lymph-node metastasis of a human tongue base cancer patient.²² The original tumor SP-C3 cells were moderately differentiated from human tongue base cancer. Cells were maintained in Dulbecco's modified eagle medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS, Moregate BioTech, Bulimba, Australia), 100 µg/ml streptomycin, and 100 units/ml penicillin (Invitrogen Corp, Carlsbad, CA, USA).

Construction of a mammalian expression vector. The mammalian expression vectors pcDNA3.1-p27^{Kip1}wt (Invitrogen) containing sense oriented human wild type p27^{Kip1} cDNA was constructed. Briefly, pcDNA3.1 (+) was digested with Kpn1 (Takara Biomedicals, Kusatsu, Japan) and BamH1 (Takara), and dephosphorylated by calf intestinal alkaline phosphate (Roche Diagnostics, Mannheim, Germany). The human wild type p27^{Kip1} cDNA fragment (0.69 kb Kpn1 and BamH1 fragment) was obtained as a generous gift from Dr. J. Massague (Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, NY). This fragment containing the human wild type p27^{Kip1} open reading frame was ligated to the prepared cloning site of pcDNA3.1 (+) by T4 DNA ligase (Takara). The direction of the ligated fragmen was confirmed by sequencing analysis with a spesific primer (p27^{Kip1}-

SQP: 5'-ATGTCAAACGTGGCGAGTGTC-3') for human p27^{Kip1} cDNA. The DNA sequence was determined by the dideoxy chain termination method, using fluorescence-labeled primers and a Thermo Sequenase Cycle sequencing kit (Amersham Pharmacia Biotech, Sweden). Electrophoresis and scanning were performed with a Shimadzu DSQ-500 DNA sequencer (Shimadzu, Kyoto, Japan).

In vivo tumorigenesis and electrotransfection. The human tongue base cancer cell line, SP-C3 cells were trypsinized, washed with PBS, and suspended in saline solution at 1×10^6 cells in 0.1 ml. Cell suspension (0.1 ml) was injected into each male nude mouse with Balb/cA Jcl-nu genetic background (Clea Japan, Inc. Tokyo, Japan) subcutan in the flank area. A pair of 1 cm diameter of disc-shaped electrodes (pinsettes-type electrode 449-10 PRG, Meiwa Shoji, Tokyo, Japan) was used to nip the tumor nodule through the skin. A series of eight electrical pulses with pulse length of 1 msec was delivered with a standard square wave electroporator BTX T820 (BTX, Inc, San Diego, CA). The voltage of 80 V/1.0 cm diameters of xenografts was used. Then, it delivered an appropriate pulse length and frequency of pulses according to previous report.^{24,25} Immediately after electrical pulsing, 20 μ g of pcDNA-neo or pcDNA3.1-p27^{Kip1} wt dissolved in 50 μ l of Tris EDTA buffer was directly injected into the tumor nodule. This electroporation and injection were performed a total of three times at 3-day intervals. Tumor volume and body weight were measured every 3 days from the time electroporation started until the mice were sacrificed. The tumor volume was determined by measuring length (L) and width (W) diameters of the tumor and calculated as $V = 0.4 \times L \times W^2$.¹³

Detection of reporter gene expression in vivo. For fluorescence microscopy, pEGFP-C3 vector (BD Bioscience Clontech) -injected tumors for 48 h were sectioned and mounted in PBS for immediate microscopy. To visualize EGFP, a Xenon arch lamp and a FITC filter were used on a Zeiss Axioskop. Images were acquired with a color CCD camera and frame-grabbing equipment at identical magnification, light intensity and amplification for each sample.¹³

Western blotting analysis. Cell lysates were prepared from the xenograft tumor tissue. Briefly, samples containing equal amounts of protein (50

μ g) were electrophoresed on a SDS-polyacrylamide gel and transferred to a nitrocellulose filter (PVDF membrane: BioRad, Hercules, CA, USA). The filters were blocked in TBS containing 5% nonfat milk powder at 37°C for 1 hour and then incubated with a 1:1000 dilution of the monoclonal antibody against p27 protein (clone 1B4, monoclonal antibody, Novocastra Laboratories, New Castle, UK) and an Amersham ECL kit (Amersham Pharmacia Biotech) as the primary antibody. Mouse antibody IgM was used as the secondary antibody (1:2000). Anti- α tubulin monoclonal antibody (Zymed laboratories, San Fransisco, CA, USA) was used for normalization of Western blot analysis.

Detection of apoptosis using colorimetric assay. Induction of apoptosis was measured using the colorimetric assay kit (caspase-3 and caspase-9; BioVision Research Product, CA, USA) according to the manufacturer's directions. Briefly, equal amounts of tissue extracts prepared from SP-C3 transfected with pcDNA3.1-p27^{Kip1} wt or pcDNA3.1-neo were incubated with the substrate (Ac-DVED-pNA or Ac-LEHD-pNA) in the assay buffer for 2 hours at 37°C. Absorbance was measured at 405 nm using a microplate reader (BioRad, USA). Each determination was performed in triplicate.

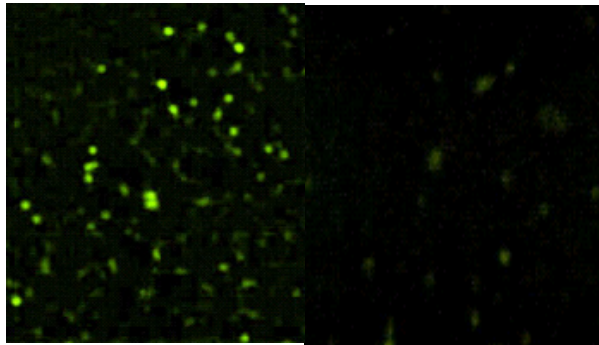
Statistical analysis. Statistical analysis was performed with a Stat Work program for Macintosh computers (Cricket Software, Philadelphia, PA, USA). Data were analyzed for statistical significance of 95% with two-way ANOVA and Student's t-test.

RESULTS

Detection of transgene expression in Xenograft. Expression of reporter gene (EGFP) after plasmid injection and electro-gene therapy in tumor tissue was assessed in fresh tissue sections by light microscopy fluorescence imaging. Very few cells were positive when only naked DNA without consecutive electroporation was injected. The combination with electroporation resulted in consistently efficient transduction of a higher number of cells with EGFP reporter gene ($P = 0.001$) (Fig. 1).

In vivo effect of p27^{Kip1} wt transfection by electroporation on tumor growth. The mean relative volume for SP-C3 xenografts treated with an injection of pcDNA3.1-p27^{Kip1} wt or pcDNA3.1 empty vector was shown in Fig. 2A, p27^{Kip1}-up-

EGFP	+	+
Electro gene therapy	-	+



2.7+0.6 70.6+2.7
Figure 1. Detection of reporter gene expression.

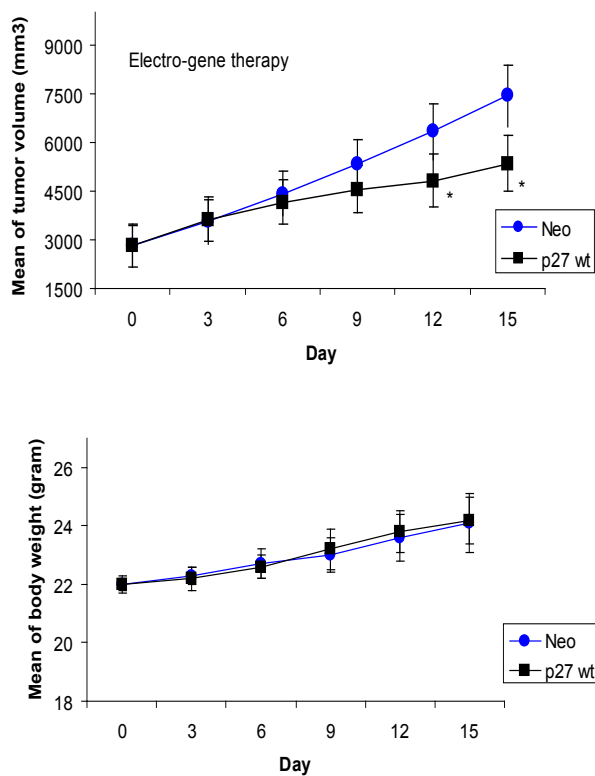


Figure 2. A. Growth of tumor formed by transfectants, B. Change of body weight in mice. * P < 0.05.

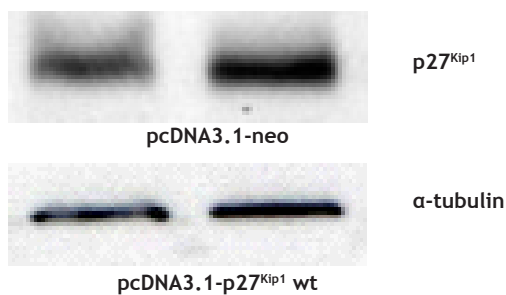


Figure 3. Expression of p27^{Kip1} and α -tubulin protein in the pcDNA3.1-neo and pcDNA3.1-p27^{Kip1} wt transfectant tissues.

regulated tumors (pcDNA3.1-p27^{Kip1} wt-injected) became much smaller than pcDNA3.1 empty vector-injected tumors (P = 0.001). Interestingly, during the experimental period, no loss of body weight was observed in each treatment group, and that no skin region including a burn also was observed (Fig. 2B).

Expression of p27^{Kip1} protein in xenografts.

To evaluate the efficiency of transfection of p27^{Kip1} gene, the expression of p27^{Kip1} protein by Western blotting was evaluated. As shown in Figure 3, up-regulated of p27^{Kip1} protein in pcDNA3.1-p27^{Kip1} wt-injected tumors was detected when compared with that in pcDNA3.1 empty vector-injected tumors. The expression of α -tubulin as an internal control was approximately the same in all of the tumors.

Proteolytic activities of caspase-3 and -9.

The activity of caspase-3 dan -9 in SP-C3-p27^{Kip1} wt or SP-C3-neo was investigated. As seen in Figure 4, SP-C3-p27^{Kip1} wt showed increased caspase-3 and -9 proteolytic activities as compared with that of SP-C3-neo. Proteolytic activities of caspase-3 in SP-C3-p27^{Kip1} wt was 2.9 fold increased compared with SP-C3-neo. Furthermore, proteolytic activities of caspase-9 in SP-C3-p27^{Kip1} wt was 1.7 fold increased (P = 0.001).

DISCUSSION

Cell membranes electro-gene therapy has been developed for the purpose of achieving highly efficient *in vitro* gene and/or drug transfer.² Interestingly, the application of electro-gene therapy to cultured cells has been well established, but the use of *in vivo* electro-gene therapy has received little attention.⁹ *In vivo* electro-gene therapy has just recently been proposed for transdermal drug delivery⁵ and for electrochemotherapy with bleomycin of superficial tumors.⁶ Recently, successful *in vivo* transfer of IL genes into muscle, and transfer of marker and therapeutic suicide genes into normal tissues and tumors have been reported.²⁶

In the present study, electro-gene therapy with naked plasmid DNA was evaluated. The wild type p27^{Kip1} gene was used as a transfection gene and was evaluated its antitumor activity in human tongue base cancer (SP-C3 cell) xenograft. The results of study demonstrated the efficiency of

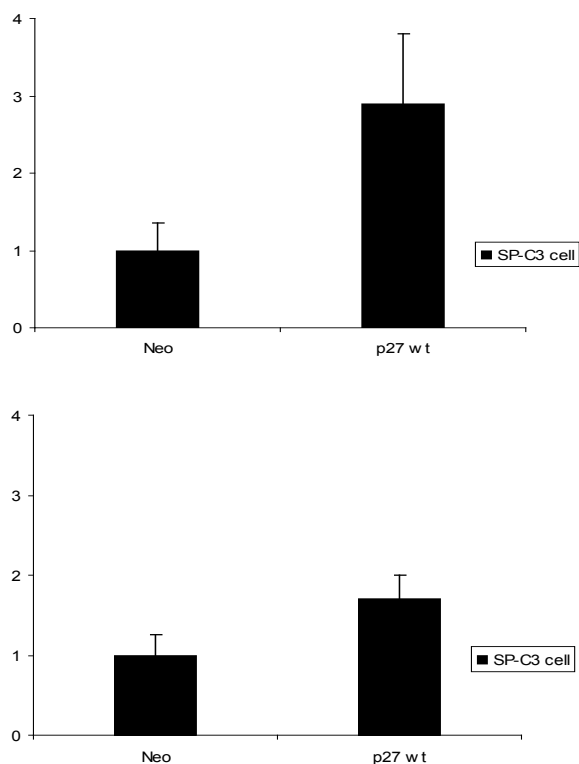


Figure 4. The activity of caspase-3 and -9 in SP-C3 transfected cells.

electro-gene therapy was thought to be about 55-70% of cells, determined by EGFP expression as shown in Figure 1. Recently, the efficiency of electro-gene therapy followed by injection of pcDNA3.1-p27^{Kip1} wt in human salivary gland cancer was about 45-65%, and in a tongue cancer SP-C1 was about 53-75%. Interestingly, the efficiency of electro-gene therapy followed by injection of 5-fluorouracil in oral cancer was still lower than of non viral plasmid injection.²⁹ Also, it was demonstrated that the transfection of wild type p27^{Kip1} gene by electro-gene therapy could induce apoptotic cell death (Fig. 4), and inhibit the growth of SP-C3 cancer xenografts (Fig. 2). Furthermore, wild type p27^{Kip1} gene by electro-gene therapy could induce the expression of p27^{Kip1} protein (Fig. 3), which has the negative regulator function in the cell cycle. Therefore, electro-transfer of plasmid DNA p27^{Kip1} wt into SP-C3 xenograft can be successfully achieved using disk-shaped electrodes. Suggesting that clinical application using this electro-gene therapy system for oral cancer may be possible in the future.

On the other hands, some disadvantages of this method should be considered. Although transfection by electro-gene therapy inhibited

the growth of SP-C3 cancer xenografts, the target area was limited to local tumors and the growth of multiple metastatic lesions cannot be target for efficient suppression. For that reason, with a view to obtaining more effective gene therapy using electroporation for head and neck cancer and oral cancer, writer plan to attempt gene transfer with several other genes and to use various anticancer agents in combination with gene transfection by this electro-gene therapy system.

In conclusion, *in vivo* electro-gene therapy following by intratumoral injection of pcDNA3.1-p27^{Kip1} wt gene has a highly antitumor activity in human tongue base cancer xenografts. It might be possible to transfer pcDNA3.1-p27^{Kip1} wt gene into human tongue base cancer xenograft. *In vivo* gene transfer method is a simple procedure and can solve some of the critical drawbacks of the present gene transfer techniques, thus providing a new strategy for gene therapy.

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