Electro-gene therapy in a human oral tongue cancer cell by intratumoral injection of pcDNA3.1-p27^{Kip1} wt

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ABSTRACT

Oral tongue cancers are characterized by a high degree of local invasion and a high rate of metastases to the cervical lymph nodes. Also, treatment options for this cancer are limited. However, a new strategy for refractory cancer, gene therapy is watched with keen interest. Recently, a novel method for high-efficiency and region-controlled in vivo gene transfer was developed by combining in vivo electro-gene therapy and intratumoral plasmid DNA injection. In the present study, a nonviral gene transfer system, in vivo electro-gene therapy in human oral tongue cancer cell, SP-C1 xenograft was examined. The aim of the study is to examine the efficiency of transfection of exogenous p27^{Kip1} gene by electroporation and the antitumor activity of p27^{Kip1} gene therapy in human oral tongue cancer xenografts using pcDNA3.1-p27^{Kip1} wild type (wt) and pcDNA3.1 empty vector with the local application of electric pulses. To evaluate this in vivo gene transfer method, the enhanced green fluorescence protein (EGFP) gene was transfected into xenografts by electroporation. The efficiency of transfection of exogenous p27^{Kip1} gene by electroporation was confirmed by Western blotting analysis. To estimate the reduction of oral tongue cancer xenografts by this method, the size of SP-C1 xenografts in nude mice after electroporation with wild type p27^{Kip1} gene was measured. The growth of tumors was markedly suppressed by wild type p27^{Kip1} gene transfection by electroporation compared with transfection of empty vector only. Moreover, histological specimens revealed apoptotic cell death was increased in wild type p27^{Kip1}-transfected tumors than empty vector. These results suggest that it is possible to transfer wild type p27^{Kip1} into human oral tongue cancer xenografts using electroporation. Wild type p27^{Kip1} has a high-potencially to suppress the growth of tumors. Finally, combination system of pcDNA3.1-p27^{Kip1} wt-injected tumor and electroporation might be used for human oral cancer.

Key words: Intratumoral, Wild type p27^{Kip1}, Human oral tongue cancer, Electroporation

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INTRODUCTION

Oral tongue cancers are characterized by a high degree of local invasion and a high rate of metastases to the cervical lymph nodes. Moreover, oral tongue cancer frequently shows local recurrence after initial treatment, probably due to microinvasion and/or micrometastasis of tumor cells at the primary site.[1] 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.[2] Furthermore, the ratio of mortality or incidence in 1980 and 1990 was 0.48 and 0.47, respectively,[3] 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.

Electro-gene therapy or electroporation has been developed for the purpose of achieving highly efficient *in vitro* gene or drug transfer.[4,5] This system provides markedly higher efficiency transfer compared with other nonviral transfer system, including cationic liposomes.[6] Electroporation has been applied to *in vivo* drug transfer for cancer treatment and clinical trial has been started.[7]

Electroporation has become more and more popular as an effective technique for introduction of foreign DNA into cells of various kinds of mammalian cells,[8,9] for investigation of gene regulation,[10] and has been demonstrated to be highly useful in transfecting human hematopoetic stem cells for gene therapy.[11] However, the transfection efficiency in mammalian cells using *in vivo* electroporation has received little attention[12] and usually is still low, typically about 0.01–1%.[13] Because electroporation is a physical method, it has a little biological side effect and is free of chemical toxicity.[10]

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.[4] Nonviral 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, assess 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.[14]

p27[Kip1] is an universal cyclin-dependent kinase inhibitor that directly inhibits the enzymatic activity of cyclin-CDK complexes, resulting in cell cycle arrest at G_1.[15] p27[Kip1] has an important prognostic factor in various malignancies. Recently, decreased expression of p27[Kip1] has been frequently detected in human cancer.[16–18] In addition, loss of p27[Kip1] has been associated with disease progression and an unfavorable outcome in several malignancies.[19] Furthermore, mice lacking the p27[Kip1] gene show an increase in body weight, thymic hypertrophy and hyperplasia of pituitary intermediate lobe adrenocorticotropic hormon cells, adrenal glands and gonadal organ.[20] Also, malignant human oral cancer cells transfection with p27[Kip1] gene leads to inhibition of proliferation, invasion and metastasis.[21,22]

In the present study, the efficiency of transfection of exogenous p27[Kip1] gene by electroporation and the antitumor activity of p27[Kip1] gene therapy in human oral tongue cancer xenografts using pcDNA3.1-p27[Kip1] wild type (wt) and pcDNA3.1 empty vector with the local application of electric pulses was evaluated.

MATERIALS AND METHODS

Cell and cell culture

SP-C1 cells were isolated from a cervical lymph-node metastasis of an oral squamous cell carcinoma patient in our laboratory.[22] The original tumor of SP-C1 cells was moderately differentiated squamous cell carcinoma of tongue, and was not invasive into muscle layer. 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 fluorescene-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).

Tumorigenesis in nude mice and electrotransfection

The oral tongue cancer cell line, SP-C1 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 back 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 100 V/1.0 cm diameters of xenografts was used. Then, it delivered an appropriate pulse length and frequency of pulses according to previous report.[8,9] Immediately after electrical pulsing, 20 μg of plasmid cDNA or pcDNA3.1-p27[Kip1] wt dissolved in 50 μl 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 $V = 0.4 \times L \times W^2$.[21]

Detection of repoter 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 pair of tumors from electroporated or non-electroporated animals, respectively.

Western blotting analysis

Cell lysates were prepared from the xenograft tumor tissue. Briefly, samples containing equal amounts of protein (50 μg) 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: 500 dilution of the monoclonal antibody against p27 protein (clone 1B4, monoclonal antibody, Novocastra Laboratories, New Castle, UK) as the primary antibody and an Amersham ECL kit (Amersham Pharmacia Biotech). Anti-α tubulin monoclonal antibody (Zymed laboratories, San Fransisco, CA, USA) was used for normalization of Western blot analysis.

TUNEL method

Apoptosis was analyzed in situ by the TUNEL technique using ApopTag (Oncor, Inc., Gaithersburg, MD, USA), labeling 3'-OH DNA ends generated by DNA

fragmentation. Mice tumors were fixed in 4% formaldehyde in PBS (–). Dewaxed paraffin sections were treated with $20\,\mu g/ml$ proteinase K in PBS (–) for 10 minutes to digest protein, then treated with 3% hydrogen peroxide in PBS for 5 minutes to quench endogenous peroxidase activity and equilibrated. TdT enzyme was applied to the cells or sections were incubated at 37ºC for 1 h. After each step, the sections were rinsed with PBS.
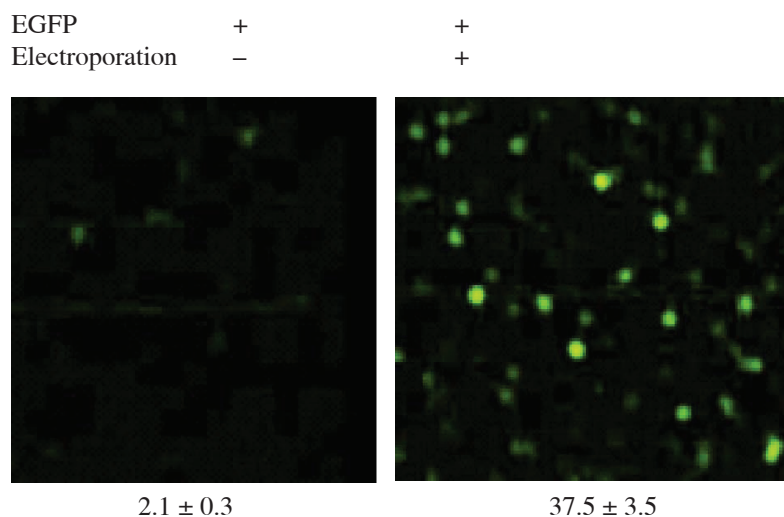
Statistical analysis

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*.
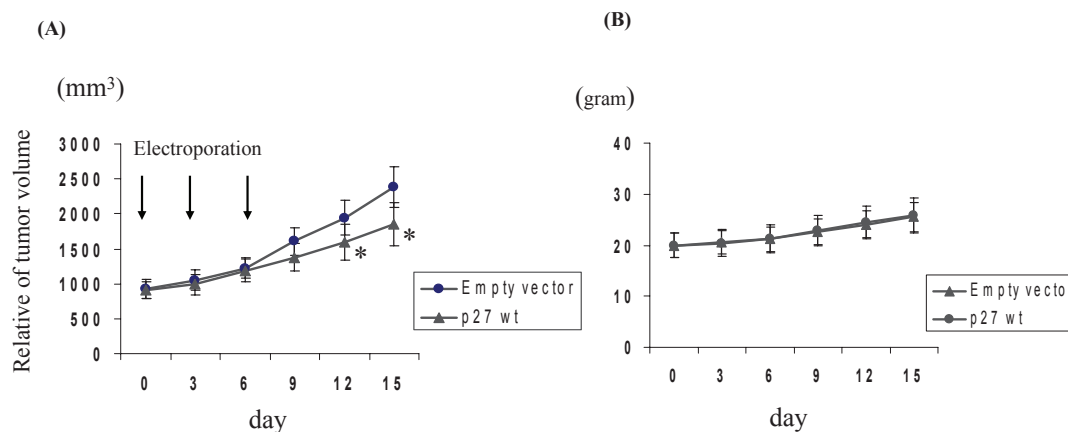
RESULTS

Detection of transgene expression in Xenograft

Expression of reporter gene (EGFP) after plasmid injection and electroporation in tumor tissue was assessed
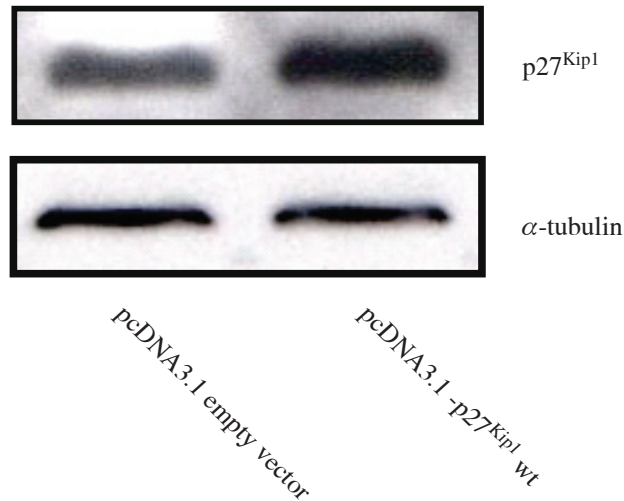
in fresh tissue sections by light microscopy fluorescence imaging. Very few cells were positive when only naked DNA without consecutive electroporation was injected. The combination with electroporation resulted in consistenly efficient transduction of a higher number of cells with EGFP reporter gene (Figure 1).

In vivo effect of p27^{Kip1} wt transfection by electroporation on tumor growth

The mean relative volume for SP-C1 xenografts treated with an injection of pcDNA3.1-p27^{Kip1} wt or pcDNA3.1 empty vector was shown in Figure 2A. p27^{Kip1}-up-regulated tumors (pcDNA3.1-p27^{Kip1} wt-injected) became much smaller than pcDNA3.1 empty vector-injected tumors ($p < 0.01$). 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 (Figure 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 Westen bloting

| EGFP | + | + |
| Electroporation | – | + |

| 2.1 ± 0.3 | 37.5 ± 3.5 |

Figure 1.   Detection of reporter gene expression.

Figure 2.   (A) Growth of tumors formed by transfectants, (B) change of body weight in mice.

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

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.

Detection of apoptosis induces by pcDNA3.1-p27^{Kip1} wt

To assess the incidence of apoptotic cell death, the internucleosomal DNA fragmentation using the TUNEL method was investigated. The TUNEL-positive cells were significantly increased in tumors electroporated with pcDNA3.1-p27^{Kip1} wt when compared with that in samples from xenografts electroporated with pcDNA3.1 empty vector (Figure 4).

DISCUSSION

Cell membranes electro-gene therapy (electroporation) has been developed for the purpose of achieving highly efficient in vitro gene and/or drug transfer.[5] Interestingly, the application of electroporation to cultured cells has been well established, but the use of in vivo electroporation has received little attention.[12] In vivo electro-gene therapy has just recently been proposed for transdermal drug delivery[23] and for electrochemotherapy with bleomycin of superficial tumors.[24] Recently, successful in vivo transfer of IL genes into muscle, and transfer of marker and therapeutic suicide genes into normal tissues and tumors has been reported.[25]

In the present study, electro-gene therapy with naked plasmid DNA was evaluated. The wild type p27^{Kip1} gene

pcDNA3.1 empty vector   pcDNA3.1-p27^{Kip1} wt
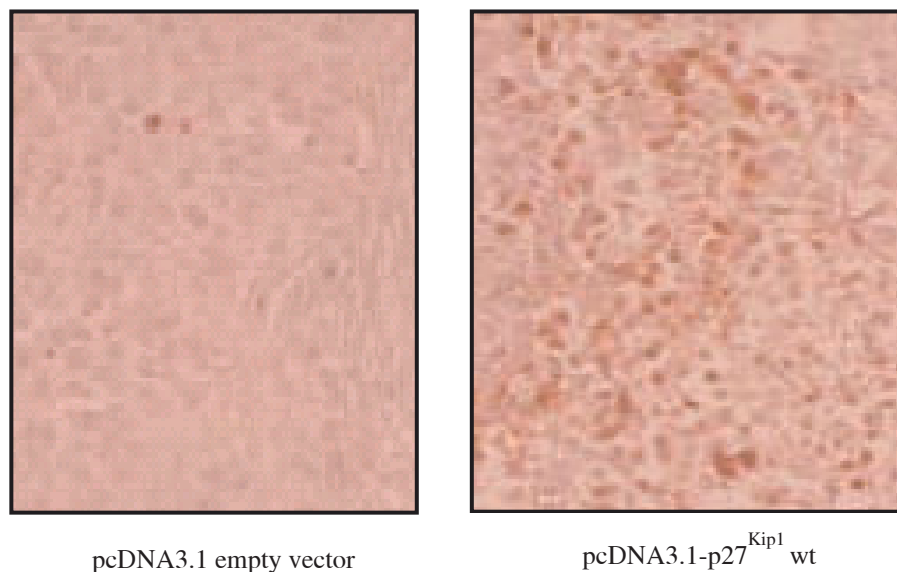
Figure 4. TUNEL, p27^{Kip1} staining of SP-C1 xenotransplants.

was used as a transfection gene and was evaluated its antitumor activity in human oral tongue cancer (SP-C1 cell) xenograft. The results of study demonstrated the efficiency of electro-gene therapy was thought to be about 40–65% of cells, determined by EGFP expression as shown in figure 1. Also, it was demonstrated that the transfection of wild type p27^{Kip1} gene by electro-gene therapy could induce apoptotic cell death (Figure 4), and inhibit the growth of oral cancer xenografts (Figure 2). Also, wild type p27^{Kip1} gene by electro-gene therapy could induce the expression of p27^{Kip1} protein (Figure 3), which has the negative regulator function in the cell cycle. Therefore, electro-transfer of plasmid DNA p27^{Kip1} wt into SP-C1 xenograft can be successfully achieved using disk-shaped electrodes. Suggesting that clinical application using this electroporation 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-C1 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 oral cancer, I 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, intratumoral injection of pcDNA3.1-p27^{Kip1} wt gene following in vivo electro-gene therapy has a highly antitumor activity in oral tongue cancer xenografts. It might be possible to transfer pcDNA3.1-p27^{Kip1} wt gene into oral 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.

ACKNOWLEDGMENTS

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