

Effect of intratumoral injection of mutant type p27^{Kip1} followed by *in vivo* electroporation on radiotherapy-resistant human oral tongue cancer xenografts

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Abstract. Oral tongue cancer is characterized by a high degree of local invasion and a high rate of metastasis to the cervical lymph nodes. Treatment options for this cancer are limited. However, gene therapy has attracted keen interest as a new strategy for refractory cancer. The aim of this study was to examine the efficiency of transfection of the exogenous p27^{Kip1} gene by electroporation and the antitumor activity of p27^{Kip1} gene therapy in radiotherapy-resistant human oral tongue cancer xenografts using mutant type (mt) pcDNA3.1-p27^{Kip1}, followed by *in vivo* electroporation. Evaluation of the *in vivo* gene transfer method was carried out by transfecting the enhanced green fluorescence protein (EGFP) gene into xenografts by electroporation. The efficiency of p27^{Kip1} gene transfection was confirmed by Western blot analysis. Estimation of the reduction in size of the B88 and B88-R-Rad tumors in mice after electroporation with the p27^{Kip1} mt gene was examined by tumorigenesis assay. The results revealed that the efficiency of transfection of B88-EGFP and B88-R-Rad-EGFP was 58.1 and 27.4%, respectively. The growth of tumors was markedly suppressed by p27^{Kip1} mt gene transfection by electroporation on B88-p27^{Kip1} mt and B88-R-Rad-p27^{Kip1} mt. Furthermore, up-regulation of p27^{Kip1} protein was detected in B88-p27^{Kip1} mt and B88-R-Rad-p27^{Kip1} mt, while inhibition of tumor size was highly increased in B88-p27^{Kip1} mt compared to B88-R-Rad-p27^{Kip1} mt. These results indicate that the intratumoral injection of pcDNA3.1-p27^{Kip1} mt with electroporation exhibited a high potential antitumor activity in human oral tongue cancer cell B88 xenografts, and a slight increase in antitumor activity in the radiotherapy-resistant human oral tongue cancer cell B88-R-Rad xenografts.

Introduction

Oral tongue cancer is characterized by a high degree of local invasion and a high rate of metastasis to the cervical lymph nodes. This cancer frequently shows local recurrence after initial treatment, probably due to microinvasion and/or micrometastasis of tumor cells at the primary site (1). Despite advances in surgery, radiotherapy and chemotherapy, the survival of patients with oral tongue cancer has not significantly improved over the past several decades. Treatment options for recurrent or refractory oral cancers are limited (2). Furthermore, the ratio of mortality to 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 has attracted keen interest.

Electroporation, also termed electric pulses or electro-gene therapy, has been developed to achieve a higher efficiency of *in vitro* gene or drug transfer (4,5). This system provides markedly higher transfer efficiency compared to other non-viral transfer systems, including cationic liposomes (6). Electric pulse has been applied to *in vivo* drug transfer for cancer treatment, and a clinical trial has been initiated (7). Electric pulse has become increasingly popular as an effective technique for introducing foreign DNA into various types of mammalian cells (8,9). This technique has been used to investigate gene regulation (10) and has been demonstrated to be highly useful in transfecting human hematopoietic stem cells for gene therapy (11). However, transfection efficiency in mammalian cells using *in vivo* electroporation has received little attention (12) and efficiency is usually quite low, typically approximately 0.01-1% (13). As a physical method, electroporation has few biological side effects and is free of chemical toxicity (10).

Many methods and techniques for *in vivo* gene transfer have been developed, and some have 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, as use of this system obviates the necessity to construct viral vectors, establish clones

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of producer cells, and assess viral titers and the presence of replication-competent helper virus, which has been known to activate passive oncogenes. The transfer procedure could be easily repeated, as 'naked' plasmid DNA has little antigenicity to the host body (14).

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 (15). p27^{Kip1} is an important prognostic factor in various types of 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 types of malignancy (19). Furthermore, mice lacking the p27^{Kip1} gene showed an increase in body weight, thymic hypertrophy and hyperplasia of pituitary intermediate lobe adrenocorticotrophic hormone cells, adrenal glands and gonadal organs (20). Malignant human oral cancer cell transfection with the p27^{Kip1} gene also leads to inhibition of proliferation, invasion and metastasis (21,22). A low level of p27^{Kip1} expression was associated with poor prognosis and high malignancy of human colon (23), breast (16), gastric (25), lung (26), prostate (27), ovarian (28), thyroid (29) and lymphatic (30) cancers. Disruption of the cell cycle regulatory activity of p27^{Kip1} is related to the aggressiveness of cancer cells (31).

Mutation of the p27^{Kip1} gene seems to be uncommon in human malignancies (32). It has been demonstrated that p27^{Kip1} is poly-ubiquitinated both *in vitro* and *in vivo*, and that p27^{Kip1} ubiquitination requires phosphorylation on threonine residue 187 (Thr 187) both *in vitro* and *in vivo* (33). We previously constructed an expression vector to express the mutant type p27^{Kip1} gene (pcDNA3.1-p27^{Kip1} mt) with a mutation of Thr-187/Pro-188 (ACGCCC) into Met-187/Ile-188 (ATGATC), which is not influenced by ubiquitin-mediated degradation (34).

In the present study, the efficiency of exogenous p27^{Kip1} gene transfection by electric pulse and the antitumor activity of the p27^{Kip1} gene in radiotherapy-resistant human oral tongue cancer xenografts using the pcDNA3.1-p27^{Kip1} mt gene with the local application of electric pulses were evaluated.

Materials and methods

Cells and cell culture. B88 cells were isolated from a cervical lymph node metastasis of an oral tongue squamous cell carcinoma patient (22). The primary tumor of B88 cells was moderately differentiated squamous cell carcinoma of the tongue, and was not invasive into the muscle layer. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS; Moregate BioTech, Bulimba, Australia), 100 µg/ml streptomycin, and 100 U/ml penicillin (Invitrogen Corp., Carlsbad, CA, USA).

***In vitro* radiotherapy.** *In vitro* B88 cells on a dish were irradiated with 0.1 Gy twice per week for 3-4 months using an X-ray irradiator (MBR-1505R2, 150 kV, 5 mA, filter 1.0-mm aluminum; Hitachi Medico, Tokyo, Japan). Gradually, cells were irradiated with 0.5 Gy twice per week for 6 months, continued with 1, 1.5 and 2 Gy. To evaluate whether the B88

cells were resistant to radiotherapy, the cells were examined by MTT and clonogenic assays.

Construction of a mammalian expression vector. The mammalian expression vector pcDNA3.1-p27^{Kip1} mt (Invitrogen) containing sense-oriented human mutant type p27^{Kip1} cDNA was constructed. Briefly, pcDNA3.1 (+) was digested with *AgeI* (Takara Biomedicals, Kusatsu, Japan) and *NheI* (Takara), and dephosphorylated by calf intestinal alkaline phosphate (Roche Diagnostics, Mannheim, Germany). The human mutant type p27^{Kip1} cDNA fragment (0.59-kbp *AgeI* and *NheI* fragment) was a generous gift from Dr Koji Harada (Department of Therapeutic Regulation for Oral Tumors, Institute of Health Biosciences, Tokushima University, Japan). This fragment, containing the human mutant 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 fragment was confirmed by sequencing analysis with a specific primer (p27^{Kip1}-SQP: 5'-ATGTCAAACGTGGCGAGTGTGTC-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).

Tumorigenesis in mice and electrotransfection. B88 and B88-R-Rad cells were collected and suspended in saline solution at 1x10⁶ cells per 0.1 ml. The cell suspension (0.1 ml) was injected subcutaneously into the flank area of each male Wistar mouse with a BALB/cA Jcl-nu genetic background (LPPT-UGM, Yogyakarta, Indonesia). A pair of 1 cm diameter 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 a pulse length of 1 msec were delivered with a standard square wave electroporator BTX T820 (BTX, Inc., San Diego, CA). A voltage of 80 V/1.0 cm diameter of the xenograft was used. Subsequently, an appropriate pulse length and frequency of pulses was delivered as described in previous reports (8,9). Immediately after electrical pulsing, 20 µg of plasmid cDNA or pcDNA3.1-p27^{Kip1} mt dissolved in 50 µl of Tris-EDTA buffer was directly injected into the tumor nodule. The procedure of electroporation and injection was 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 (V) was determined by measuring the length (L) and width (W) of the tumors, and was calculated using the formula: $V = 0.4 \times L \times W^2$ (21).

Detection of reporter gene expression *in vivo*. Detection of reporter gene expression *in vivo* in pEGFP-C3 vector (BD Bioscience Clontech)-injected tumors (B88 and B88-R-Rad) was carried out at 48 h by sectioning and mounting the tumors in PBS for immediate microscopy. A Xenon arch lamp and an FITC filter were used on a Zeiss Axioskop microscope to visualize enhanced green fluorescence protein (EGFP). Images were captured with a color CCD camera and frame-grabbing equipment at an identical magnification, light intensity and

amplification for each sample pair of tumors from the electroporated and non-electroporated animals, respectively.

Western blot analysis. Cell lysates were prepared from the xenograft B88 and B88-R-Rad 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 h, and then incubated with a 1:500 dilution of monoclonal antibody against the p27 protein (clone 1B4, monoclonal antibody; Novocastra Laboratories, New Castle, UK) as the primary antibody using the Amersham ECL kit (Amersham Pharmacia Biotech). Anti- α -tubulin monoclonal antibody (Zymed Laboratories, San Francisco, CA, USA) was used for normalization of the Western blot analysis.

Statistical analysis. Statistical differences between the means for the different groups were evaluated with Stat View 4.5 (version 5.0J, SAS Institute Inc, Cary, NC, USA) using one-way ANOVA and a t-test. The significance level was set at 5% for each analysis.

Ethics. This study was approved by the Ethics Committee of the Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia (no. KE/FK/166/EC/2008).

Results

Examination of cell resistance using MTT and clonogenic assays. The radiotherapy resistance of the B88-R-Rad cells was examined by the MTT assay. Relative cell number was evaluated by comparing the absorbance in each cell. No significant differences in cell number were noted between the B88-R-Rad-no radiation and B88-R-Rad-2 Gy cells. However, the cell growth of B88 cells treated with X-ray irradiation (2 Gy) was significantly suppressed as compared to that of the non-treated B88 cells ($P<0.05$) (Fig. 1). Furthermore, the growth of cell cloning of the B88 cells treated with 2 Gy X-ray irradiation was markedly suppressed as compared to that of the non-treated B88 cells. Conversely, the growth of the B88-R-Rad-irradiated and B88-R-Rad-no irradiated cells was approximately the same in all of the observed cells (Fig. 2).

Expression of p27^{Kip1} protein in the xenografts. To evaluate the efficiency of p27^{Kip1} gene transfection, the expression of p27^{Kip1} protein by Western blotting was evaluated. As shown in Fig. 3, up-regulation of p27^{Kip1} protein in the B88-pcDNA3.1-p27^{Kip1} mt- and B88-R-Rad-pcDNA3.1-p27^{Kip1} mt-injected tumors was detected and compared with that in the pcDNA3.1 empty vector-injected tumors. Expression of p27^{Kip1} protein was slightly increased in the B88-pcDNA3.1-p27^{Kip1} mt-injected tumors compared to that in the B88-R-Rad-pcDNA3.1-p27^{Kip1} mt-injected tumors. Therefore, the expression of α -tubulin as an internal control was approximately the same in each tumor observed.

Detection of transgene expression in the xenografts. Detection of reporter gene EGFP after plasmid injection and electropo-

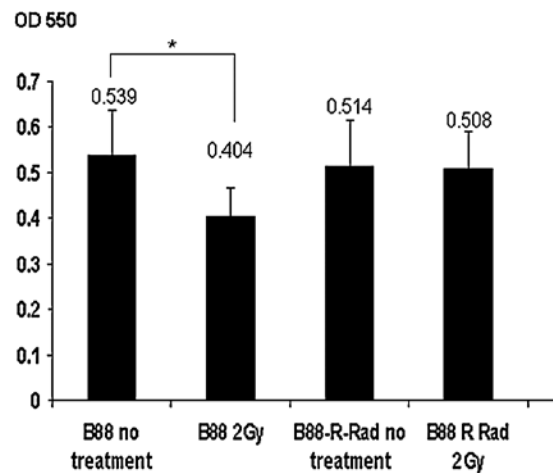


Figure 1. Growth of B88 and B88-R-Rad cells with or without radiation *in vitro*. Relative cell number was evaluated by the MTT assay at 48 h. * $P<0.05$.

ration in the tumor tissues was conducted in fresh tissue sections by microscopic fluorescence imaging. Few EGFP-positive cells were noted in the B88-no treated ($1.2\pm0.3\%$) and B88-R-Rad-no treated ($1.8\pm0.2\%$) cells when only naked DNA without consecutive electric pulse was injected. The combination treatment with electric pulse resulted in consistently efficient transduction of a higher number of B88-treated ($58.1\pm5.2\%$) and B88-R-Rad-treated ($27.4\pm4.8\%$) cells with the EGFP reporter gene (Fig. 4).

Analysis of tumorigenesis. Analysis of tumorigenesis was conducted in the flank area of the Wistar mice with the BALB/cA Jcl-nu genetic background. As shown in Fig. 5, the mean relative tumor volume of the B88-p27^{Kip1} mt and B88-R-Rad-p27^{Kip1} mt xenografts after electric pulse was significantly decreased at day 12 and 15 compared to the B88-neo or B88-R-Rad-neo xenografts, respectively ($P<0.05$). However, the antitumor activity of p27^{Kip1} mt was slightly stronger in the B88 tumor nodules than in the B88-R-Rad tumor nodules ($P<0.05$). Notably, during the experimental period, no decrease in body weight was observed in any of the treatment groups, and no burning of the skin region was noted.

Discussion

It is currently believed that the loss of normal cell cycle control plays a crucial role in the genesis of most types of cancer. The cell cycle is modulated by the interaction of multiple cell cycle molecules, including cyclins, cyclin-activating kinase (CAK), CDK and cyclin-dependent kinases inhibitors (CDI). In addition to its role as a CDI, p27^{Kip1} is a putative tumor suppressor gene (20), a regulator of drug resistance in solid tumors (35), and a promoter of apoptosis (24). Moreover, p27^{Kip1} acts as a safeguard against inflammatory injury (36) and plays a role in cell differentiation (37). It has been reported that mutant type p27^{Kip1} displayed higher antitumor effects than the wild-type in lung cancer cells. In p27 mt, Thr-187/Pro-188 was mutated into Met-187/Ile-188 to prevent the phosphorylation of Thr-187, which is the main mechanism of p27^{Kip1} degradation (38). Similarly, the growth inhibitory effect of wild-type

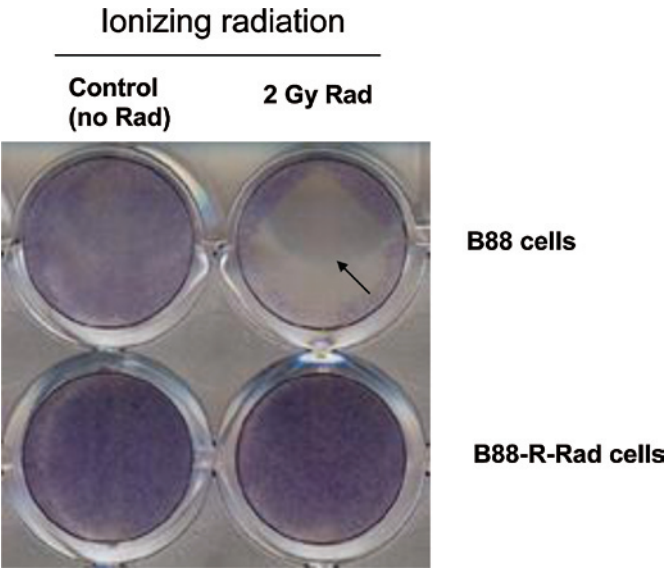


Figure 2. Growth of B88 and B88-R-Rad cells with or without ionizing radiation *in vitro*. Cell growth was evaluated by clonogenic assay at 72 h. Black arrow, area of inhibition.

p27^{Kip1} gene transfer on a head and neck carcinoma cell line (39) and the growth inhibitory effect of mutant p27^{Kip1} gene transfer on head and neck cancer cells, including oral cancer cell lines, have been reported (34,40).

In the present study, the efficiency of exogenous p27^{Kip1} gene transfection by local electric pulse and the antitumor activity of the p27^{Kip1} gene in radiotherapy-resistant human oral tongue cancer xenografts determined using the pcDNA3.1-p27^{Kip1} mt gene followed by electroporation were evaluated. An expression vector containing sense-oriented human p27^{Kip1}

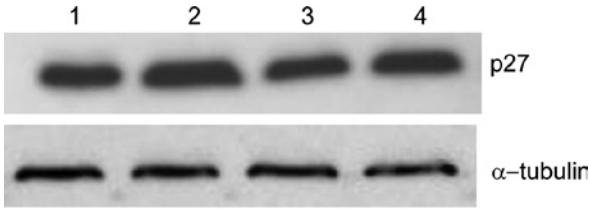


Figure 3. Expression of p27^{Kip1} and α-tubulin protein in B88-pcDNA3.1-neo, B88-pcDNA3.1-p27^{Kip1} mt, B88-R-Rad-pcDNA3.1-neo, and B88-R-Rad-pcDNA3.1-p27^{Kip1} mt. Lane 1, B88-neo; 2, B88-p27 mt; 3, B88-R-Rad; 4, B88-R-Rad-p27 mt.

cDNA with pcDNA3.1 was constructed. B88 and B88-R-Rad cells were transfected with the sense expression vector to regulate the expression of the p27 gene. MTT assay analysis indicated no difference in growth between the B88-R-Rad-no radiation and B88-R-Rad 2 Gy tumors. However, the growth of B88 cells treated with 2 Gy irradiation was significantly suppressed as compared to that of the non-treated B88 cells (Fig. 1). These data suggest that the B88-R-Rad cell line was markedly resistant to radiotherapy. Furthermore, a clonogenic assay was evaluated. The growth of cell cloning of the B88 cells treated with 2 Gy radiation was markedly suppressed as compared to that of the non-treated B88 cells. Conversely, the growth of B88-R-Rad-radiated and B88-R-Rad-no radiated cells was approximately the same in all of the observed cells (Fig. 2). Khaled *et al* (42) reported that overexpression of cyclin B1 in head and neck cancer cells is capable of inducing resistance to radiotherapy (RT), and that cyclin B1 may serve as an indicator of the risk of locoregional recurrence and metastasis in patients with HNSCC receiving RT. Moreover, Western blot analysis demonstrated the overexpres-

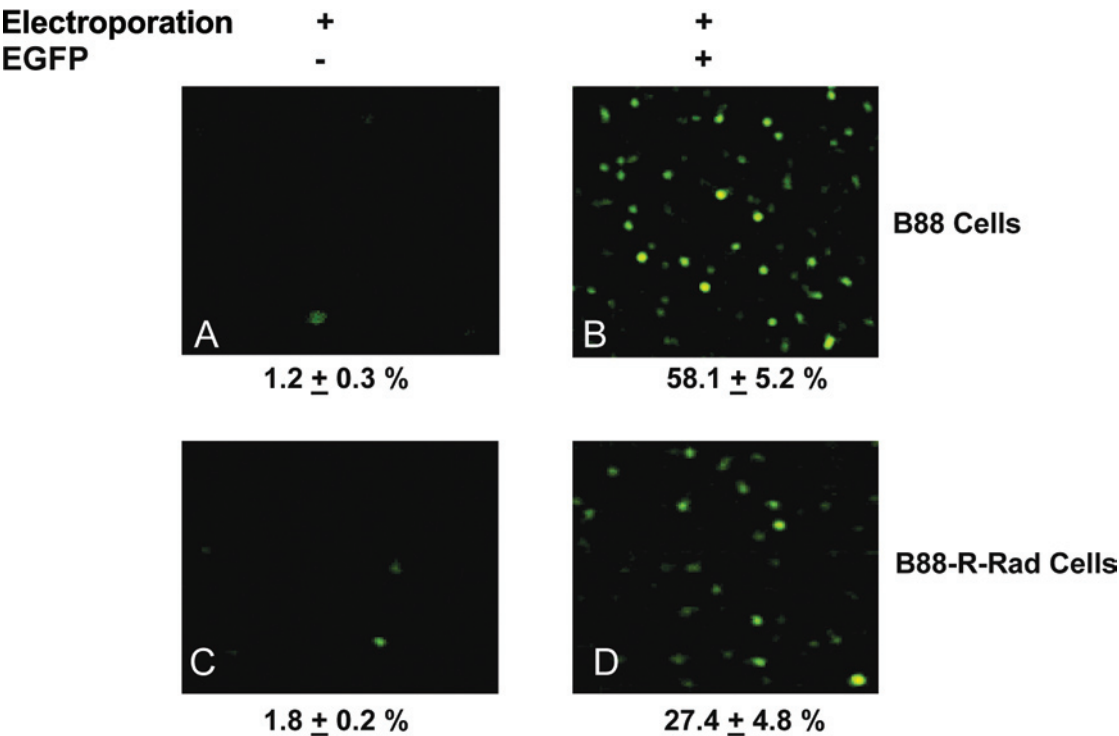


Figure 4. Detection of reporter gene expression using EGFP followed by electroporation on B88-pcDNA3.1-neo (A), B88-pcDNA3.1-EGFP (B), B88-R-Rad-pcDNA3.1-neo (C), and B88-R-Rad-pcDNA3.1-EGFP cells (D).

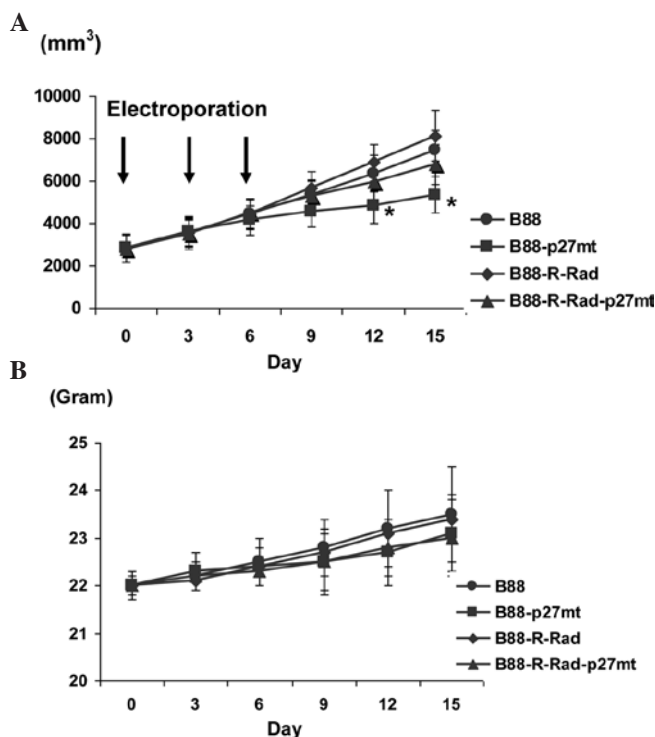


Figure 5. (A) Inhibition of tumorigenesis of B88 and B88-R-Rad cancer cells after injection of intratumoral pcDNA3.1-p27^{Kip1} mt or pcDNA3.1-neo followed by electroporation. (B) Change in body weight of the mice. *P<0.05.

sion of p27^{Kip1} protein in the B88-pcDNA3.1-p27^{Kip1} mt- and B88-R-Rad-pcDNA3.1-p27^{Kip1} mt-injected tumors. However, the expression of p27^{Kip1} protein was markedly increased in the B88-pcDNA3.1-p27^{Kip1} mt-injected tumors compared to the B88-R-Rad-pcDNA3.1-p27^{Kip1} mt-injected tumors (Fig. 3). These data indicate that the p27^{Kip1} protein in the B88-pcDNA3.1-p27^{Kip1} mt-injected tumors was more resistant to degradation by ubiquitin-proteasome and more stable than in the B88-R-Rad-pcDNA3.1-p27^{Kip1} mt-injected tumors. A recent study reported that transfection of pcDNA3.1-p27^{Kip1} mt in head and neck cancer cells induced a strong growth inhibition of the xenografted tumors, and that stability of the p27^{Kip1} protein improved therapeutic benefits in patients with head and neck cancer (40).

In the present study, we demonstrated that up-regulation of p27^{Kip1} protein exerted a growth inhibitory effect, while down-regulation of p27^{Kip1} protein enhanced the growth of cancer cells. A previous study reported that p27^{Kip1} protein levels may be controlled by the level of proteasomal proteolysis mediated by ubiquitin (41), and the process of p27^{Kip1} degradation by ubiquitin was detected in the nucleus and cytoplasm (32,40).

The combination of an intratumoral injection of the EGFP reporter gene with local electric pulse resulted in consistently efficient transduction of a higher number of B88 and B88-R-Rad cells. However, the efficient transduction of B88 cells was greatly increased compared to that in the B88-R-Rad cells (Fig. 4). The same result was revealed upon Western blot analysis. In the case of the *in vivo* tumorigenesis assay, up-regulation of p27^{Kip1} protein exerted a growth inhibitory effect. As expected from its stronger p27^{Kip1} protein expres-

sion, a significant suppression of tumor growth was detected in B88-p27^{Kip1} mt and B88-R-Rad-p27^{Kip1} mt. Notably, higher anti-tumorigenic effects were noted in B88-p27^{Kip1} mt than in B88-R-Rad-p27^{Kip1} mt (Fig. 5). Park *et al* (38) reported that mutant type p27^{Kip1} displays higher antitumor effects than wild-type p27^{Kip1} in lung cancer cells. Other data have indicated that no mice demonstrated metastasis of the cervical lymph nodes and distant metastasis in p27^{Kip1} up-regulated transfectants. Furthermore, during the experimental period, no decrease in body weight was observed in the treated mice, and no burning of the skin region was noted. From the findings described above, we suggest that p27^{Kip1} mt exerts an antitumor effect in B88 and B88-R-Rad cells. This effect was markedly increased in B88-p27^{Kip1} mt compared to B88-R-Rad-p27^{Kip1} mt.

In conclusion, intratumoral injection of pcDNA3.1-p27^{Kip1} mt followed by electroporation exhibits high potential antitumor activity in human oral tongue cancer cell B88 xenografts, while this procedure exhibits a slightly increased antitumor activity in radiotherapy-resistant human oral tongue cancer cell B88-R-Rad xenografts.

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