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Research Report

Antitumor activity of intratumoral injection of pcDNA3.1-p27^{Kip1}mt followed by in vivo electroporation in a malignant Burkitt's lymphoma cell xenograft

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ABSTRACT

Background: *Human malignant Burkitt's lymphomas are an uncommon type of Non-Hodgkin Lymphoma commonly affects in children. It is a highly aggressive type of B-cell lymphoma. Treatment for this malignant are still 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 electroporation and plasmid cDNA. In the present study, a non-viral gene transfer system, in vivo electroporation in human malignant Burkitt's lymphoma (Raji) cell xenograft was investigated.* ***Purpose:*** *The purpose of this study was to evaluate p27^{Kip1} gene therapy in Raji cell xenografts using pcDNA3.1-p27^{Kip1} mutant type (mt) and pcDNA3.1 empty vector (neo) with the local application of electric pulses.* ***Methods:*** *True experimental study using post-intervention with control group design was performed in this study. Material sample was obtained from integrated research laboratory at faculty of dentistry, Universitas Gadjah Mada, Yogyakarta. The efficiency of transfection of exogenous p27^{Kip1} gene by electroporation was confirmed by Western bloting analysis. To evaluate the reduction of malignant Burkitt's lymphoma cell xenografts by this method, the volume of Raji cell xenografts in mice after electroporation with p27^{Kip1} mt or neo gene was measured.* **Results:** *Up-regulation of p27^{Kip1} protein was detected in pcDNA3.1-p27^{Kip1} mt. Furthermore, the growth of tumors was markedly suppressed by p27^{Kip1} mt gene transfection compared with transfection of neo.* ***Conclusion:*** *Injection of pcDNA3.1-p27^{Kip1} mt gene followed by in vivo electroporation has a high-potentially to suppress the growth of malignant Burkitt's lymphoma cells. Furthermore, combination system of pcDNA3.1-p27^{Kip1} mt-injected tumor and electroporation might be used for human oral cancer.*

Key words: *Mutant type p27^{Kip1}, human malignant Burkitt's lymphoma, electroporation*

ABSTRAK

Latar belakang: *Limfoma Burkitt's maligna banyak terjadi pada anak-anak dan merupakan jenis yang langka dari limfoma Non-Hodgkin (NHL). Limfoma Burkitt's maligna adalah tipe yang sangat agresif dari limfoma sel B. Perawatan penyakit ini masih sangat terbatas, walaupun demikian strategi baru perawatan kanker menggunakan terapi gen menjadi pusat perhatian. Suatu metode baru transfer gen untuk meningkatkan efisiensi dan kontrol area telah dikembangkan dengan mengkombinasi elektroporasi in vivo dan plasmid cDNA. Pada penelitian ini, telah diteliti sistim transfer gen non-virus dengan elektroporasi in vivo terhadap xenograft sel limfoma Burkitt's maligna (sel Raji).* ***Tujuan:*** *Tujuan dari penelitian ini adalah untuk mengevaluasi terapi gen p27^{Kip1} terhadap xenograft sel Raji menggunakan pcDNA3.1-p27^{Kip1} mutant type (mt) dan pcDNA3.1 empty vector (neo) dengan aplikasi lokal elektroporasi.* ***Metode****: Jenis penelitian yang digunakan adalah eksperimen murni memakai rancangan pasca intervensi dengan kelompok kontrol. Sampel dan bahan penelitian didapat dari laboratorium riset terpadu, Fakultas Kedokteran Gigi, Universitas Gadjah Mada, Yogyakarta. Efisiensi transfeksi gen p27^{Kip1} eksogen dengan elektroporasi dilakukan dengan analisis Western bloting. Untuk mengevaluasi hambatan xenograft sel limfoma Burkitt's maligna dengan metode elektroporasi, dilakukan pengukuran volume xenograft sel Raji pada tikus pasca elektroporasi dan injeksi gen p27^{Kip1} mt atau neo.* ***Hasil:*** *Peningkatan regulasi protein p27^{Kip1} terdeteksi pada gen pcDNA3.1-p27^{Kip1} mt. Selanjutnya, pertumbuhan tumor secara signifikan terhambat oleh transfeksi gen p27^{Kip1} mt dibandingkan dengan transfeksi neo.* ***Kesimpulan:*** *Injeksi gen pcDNA3.1-p27^{Kip1} mt disertai elektroporasi in vivo mempunyai potensi yang kuat menghambat pertumbuhan*

sel limfoma Burkitt's maligna. Kombinasi sistim injeksi tumor menggunakan gen pcDNA3.1-p27^{Kip1} mt dan elektroporasi kemungkinan dapat digunakan untuk terapi kanker oral.

***Kata kunci**: p27^{Kip1} mt, limfoma Burkitt's maligna, elektroporasi*

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INTRODUCTION

Electroporation (electro-gene therapy or electric pulse) has been developed for the purpose of achieving highly efficient in vitro gene or drug transfer.[1] This system provides markedly higher efficiency transfer compared with other non-viral transfer system, including cationic liposome.[2] Electroporation has been applied to in vivo drug transfer for cancer treatment and clinical trial has been started.[3] Electroporation has become more and more popular as an effective technique for introduction of foreign DNA into cells of various kinds of mammalian cells,[4] for investigation of gene regulation,[5] and has been demonstrated to be highly useful in transfecting human hematopoietic stem cells for gene therapy.[6] However, the transfection efficiency in mammalian cells using in vivo electroporation has received little attention[7] and usually is still low, typically about 0.01-1%.[8] Because electroporation is a physical method, it has a little biological side effect and is free of chemical toxicity.[5]

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, assessed 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.[9]

Burkitt's lymphoma (BL) is a rare, highly aggressive lymphoma. It is a tumour of the immune system with variable range of incidence depending on age, geographic location, race and Epstein–Barr virus (EBV) exposure. BL is classified as a type of non-Hodgkin's lymphoma (NHL) of monoclonal small, non-cleaved B-cell lymphocytes, which is subclassified as either endemic or non-endemic.[10] This is one of the most rapidly growing paediatric tumours that require prompt diagnosis before initiation of a specific treatment.[11] Although the clinical features of endemic and non-endemic BL are dissimilar, the characteristics of the tumour cells and prognostic factors are similar.[12] According to their clinical and cytological characteristics, Burkitt's lymphoma is classified into low, medium and high grades of malignancy. High-grade Burkitt's lymphoma affects mainly young people, while low-grade malignancies are more frequent in advanced age.[10] The majority of lymphomas arise in lymphoid tissue, especially the cervical nodes (80% of all childhood neoplasm), and only 20% arise at extranodal sites.[12] The commonest tumour was seen in children 3–10 years of age,[11] and Epstein-Barr virus has been implicated in its etiology. A number of studies have assessed clinical and treatments of BL and contradictory results have been reported.[13]

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.[14] p27^{Kip1} has an important prognostic factor in various malignancies. Recently, decreased expression of p27^{Kip1} has been frequently detected in human cancer.[15-17] In addition, loss of p27^{Kip1} has been associated with disease progression and an unfavorable outcome in several malignancies.[18] 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.[19] Also, malignant human oral cancer cells transfection with p27^{Kip1} gene leads to inhibition of proliferation, invasion and metastasis.[20,21]

In the present study, the antitumor activity of p27^{Kip1} gene therapy in human malignant Burkitt's lymphoma (Raji) cell xenografts using pcDNA3.1-p27^{Kip1} mutant type (mt) and pcDNA3.1 empty vector (neo) with the local application of electric pulses was evaluated.

MATERIALS AND METHODS

Raji cells were obtained from the Integrated Research Laboratory, Faculty of Dentistry, Gadjah Mada University, Yogyakarta. 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).

The mammalian expression vectors pcDNA3.1-p27^{Kip1} mt containing sense oriented human mutant type p27^{Kip1} cDNA was constructed. Briefly, pcDNA3.1 (+) was digested with Age1 (Takara Biomedicals, Kusatsu, Japan) and Nhe1 (Takara), and dephosphorylated by calf intestinal alkaline phosphate (Roche Diagnostics, Mannheim, Germany). The human mutant type p27^{Kip1} cDNA fragment (0.59 kb AgeI and Nhe1 fragment) was obtained as a generous gift from Dr. J Massague (Howard Hughes Medical Institute,

Memorial Sloan-Kettering Cancer Center, NY) and Dr. K Harada (Department of Therapeutic Regulation for Oral tumor, Institute of Health Bioscience, 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 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). However, sequencing data is not shown in this article.

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 37^0C 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) and an Amersham ECL kit (Amersham Pharmacia Biotech) as the primary antibody. Mouse antibody IgM was used as the secondary antibody. Anti-α tubulin monoclonal antibody (Zymed laboratories, San Fransisco, CA, USA) was used for normalization of Western blot analysis.

The Raji cells were trypsinized, washed with PBS, and suspended in saline solution at 1 x 10^6 cells in 0.1 ml. Cell suspension (0.1 ml) was injected into each male Wistar mouse with Balb/cA genetic background (LPPT UGM, Yogyakarta, Indonesia) 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 standar 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.[4] Immediately after electrical pulsing, 20 μg of pcDNA-neo or pcDNA3.1-$p27^{Kip1}$ mt 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 $V = 0.4 \times L \times W^2$.

Statistical analysis was performed with a Stat Work program for Macintosh computers (Cricket Software, Philadelphia, PA, USA). In vivo tumor volume data in tumorigenesis assay were analyzed for statistical significance of 95% with two way ANOVA followed by Post-hoc LSD.

RESULTS

To evaluate the efficiency of transfection of $p27^{Kip1}$ gene, the expression of $p27^{Kip1}$ protein by Western blotting was evaluated. Equal amounts of each transfected cell protein (50 μg) were electrophoresed on a SDS-polyacrylamide gel and transferred to a nitrocellulose filter. Up-regulated of $p27^{Kip1}$ protein in pcDNA3.1-$p27^{Kip1}$ mt-injected tumors was detected when compared with that in pcDNA3.1 empty vector (neo)-injected tumors. However, the expression of α-tubulin as an internal control was approximately the same in all of the tumors (Figure 1).

The mean relative volume for Raji xenografts treated with an injection of pcDNA3.1-$p27^{Kip1}$ mt or pcDNA3.1 empty vector was shown in Figure 2. pcDNA3.1-$p27^{Kip1}$ mt-injected became much smaller than pcDNA3.1 empty vector-injected tumors, and $p27^{Kip1}$-up-regulated tumors revealed significantly suppressed the tumor volume compared with that of neo ($p<0.01$). 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.

DISCUSSION

Lack of detectable expression of $p27^{kip1}$ cyclin dependent kinase inhibitor has previously been correlated with high degree of malignancy in human cancers include breast, colorectal, gastric and small cell lung carcinomas. Furthermore, we were demonstrated that an inverse correlation between $p27^{kip1}$ expression and tumor malignancy in oral cancer.[20,21] In the present study, antitumor activity of intratumoral injection of pcDNA3.1-$p27^{Kip1}$ mutant type followed by electroporation in a human malignant Burkitt's lymphoma cell xenograft was examined. The mutant type $p27^{Kip1}$ gene was used as a transfection gene and was evaluated its antitumor activity in human malignant Burkitt's lymphoma cell (Raji cell) xenograft. The results of study demonstrated the transfection of mutant type $p27^{Kip1}$ gene by electroporation could induce the expression of $p27^{Kip1}$ protein (Figure 1), which has the negative regulator function in the cell cycle. Accumulation of $p27^{Kip1}$ protein in human malignant Burkitt's lymphoma cell was marked the good prognosis. The same result was reported by Barnouin *et al*.,[22] that $p27^{Kip1}$ has the antiproliferative function in Burkitt's lymphoma cell in vitro. Therefore, mutant type $p27^{Kip1}$ gene was markedly suppressed the growth of Raji cancer xenografts through tumorigenesis analysis (Figure 2A). Also, during the

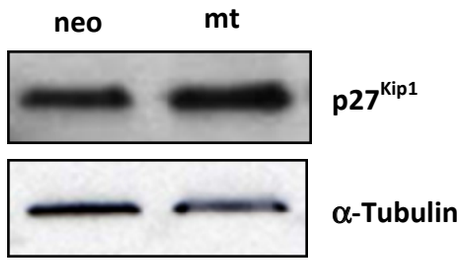

Figure 1. Western blotting analysis. Expression of $p27^{Kip1}$ and α-Tubulin protein in human malignant Burkitt's lymphoma cell transfected with pcDNA3.1-$p27^{Kip1}$ mt or empty vector.

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). Supriatno *et al.,*[23] reported that degradation of $p27^{Kip1}$ can be promoted by phosphorylation of Skp2 and Thr-187. Inversely, mutant type $p27^{Kip1}$ was originally from mutation on Thr-187/Pro-188 (ACGCCC) to Met-187/Ile-188 (ATGATC) is not influenced by ubiquitin-mediated degradation. Met-187/Ile-188 (ATGATC) is resistance to degradation.[23] Increasing $p27^{Kip1}$ protein in cancer tissues is associated with low aggressive of cancer cell and good prognosis.[23] Next, electro-transfer of plasmid cDNA $p27^{Kip1}$ mt into Burkitt's lymphoma cell xenograft can be successfully

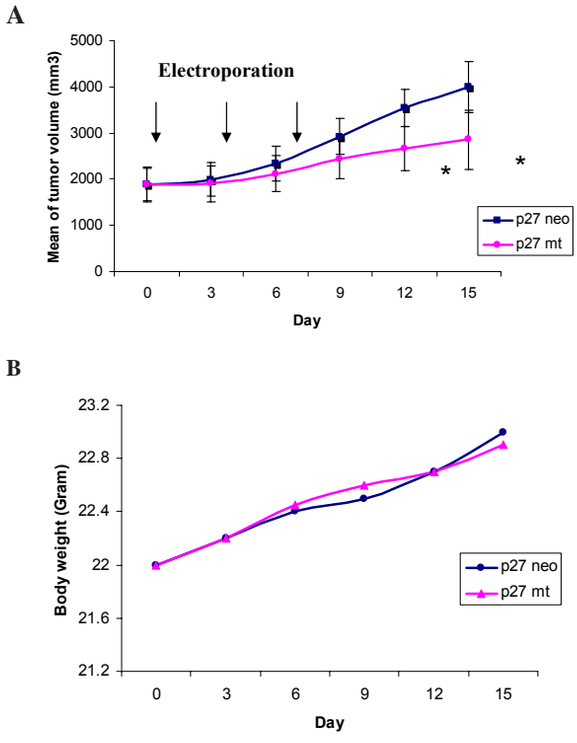

Figure 2. A) Tumorigenesis suppression of human malignant Burkitt's lymphoma (Raji) cell after injection of pcDNA3.1-$p27^{Kip1}$ mt or empty vector (neo) followed by in vivo electroporation. * $p < 0.05$; B) Change of body weight. *$p < 0.05$.

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 electroporation inhibited the growth of Raji cell 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, we plan to attempt gene transfer with several other genes and to use various anticancer agents in combination with gene transfection by this electroporation system.

In conclusion, injection of pcDNA3.1-$p27^{Kip1}$ mt gene following in vivo electroporation has a highly antitumor activity in human malignant Burkitt's lymphoma cell xenografts. It might be possible to transfer pcDNA3.1-$p27^{Kip1}$ mt gene into human malignant Burkitt's lymphoma cell 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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