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In vivo gene therapy with plasmid DNA-tuberous sclerosis complex-2 (TSC-2) in an oral squamous cell carcinoma

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ABSTRACT

Supriatno - ***In vivo gene therapy with plasmid DNA-Tuberous sclerosis complex-2 (TSC-2) in an oral squamous cell carcinoma***

Background: Tuberous sclerosis complex (TSC) is an inherited syndrome in which affected individuals are at increased risk for developing benign tumors. Mutation of the TSC-2 gene encoding tuberin on chromosome 16p13.3 give rise to the clinical disorder of tuberous sclerosis characterized by the development of hamartomas.
Objectives: In the present study, I conducted to examine whether overexpression of TSC-2 can affect the growth of human oral squamous cell carcinoma (OSCC) which have different expression level of $p27^{Kip1}$.
Methods: I constructed an expression vector containing sense-oriented rat TSC-2 cDNA with pcDNA3.1 (Invitrogen), and transfected to oral squamous cell carcinoma (B88) to regulate the expression of TSC-2 gene in each transfectant. Western blot analysis was carried out to determine the protein level of TSC-2. Therefore, Tumor volume and body weight of nude mice was examined using tumorigenicity assay.
Results: Overexpression of TSC-2 exerted the growth inhibitory effect of oral squamous cell carcinoma. Sense-oriented B88-TSC-2 cancer cells have high expression of $p27^{Kip1}$. Moreover, tumor induced by TSC-2 up-regulated transfectans become much smaller than those of control cells.
Conclusion: overexpression of TSC-2 may exert the antitumor effect on oral squamous cell carcinoma through $p27^{Kip1}$ induction.

Key word: TSC-2 - transfection - oral squamous cell carcinoma - pcDNA3.1 - mutation.

ABSTRAK

Supriatno - ***Terapi gen menggunakan plasmid DNA-Tuberous sclerosis complex-2 (TSC-2) pada sel skuamos karsinoma rongga mulut in vivo***

Latar belakang: *Tuberous sclerosis complex* (TSC), suatu sindrom bawaan yang mempengaruhi individu terhadap tingginya resiko pertumbuhan tumor jinak. Mutasi gen TSC-2 atau disebut tuberin, pada kromosom 16p13.3 menimbulkan kelainan klinik *tuberous sclerosis* yang ditandai dengan berkembangnya hamartoma.
Tujuan: Penelitian ini menguji tingginya ekspresi TSC-2 mempengaruhi pertumbuhan sel skuamos karsinoma rongga mulut manusia (OSCC) yang mempunyai perbedaan ekspresi $p27^{Kip1}$.
Metode: Ekspresi vector *sense-oriented rat* TSC-2 cDNA dikonstruksi menggunakan pcDNA3.1 (Invitrogen), kemudian ditransfeksikan pada sel skuamos karsinoma (B88) untuk mengatur ekspresi gen TSC-2 pada setiap sel transfektan. Untuk menentukan tingkat protein TSC-2 dilakukan analisis Western blot. Volume tumor dan berat badan tikus nude dilakukan dengan uji tumorigenisitas.
Hasil: Tingginya ekspresi TSC-2 menunjukkan hambatan pertumbuhan sel skuamos karsinoma. Sel B88-TSC-2 (*sense-oriented*) mempunyai ekspresi $p27^{Kip1}$ yang tinggi. Volume tumor pada tikus nude yang ditransfeksi dengan TSC-2 (B88-TSC-2) menunjukkan volume tumor yang lebih kecil dibandingkan kontrol.
Kesimpulan: Peningkatan ekspresi TSC-2 mempunyai efek antitumor terhadap sel skuamos karsinoma rongga mulut melalui induksi protein $p27^{Kip1}$

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INTRODUCTION

Tuberous sclerosis complex (TSC) is an inherited syndrome in which affected individuals are at increased risk for developing benign tumors included hamartomas, rhabdomyomas, angiofibromas and fibromas[1]. Manifestations in the central nervous system include mental retardation, autism and seizures[2]. TSC is an autosomal dominant tumor suppressor gene disorder affecting 1 in 6000 live births. TSC presents variably in multiple organs, including the brain, eye, skin, kidney, heart[3] and oral[4]. Linkage analysis of TSC resulted in the identification of two distinct genetic loci on chromosome 9 and 16 (5). These genes are TSC-1 (9q34) and TSC-2 (16q13), respectively[6]. Germline TSC-1 and TSC-2 mutation appear to be inactivating and loss of heterozygosity at either the TSC-1 and TSC-2 region occurs in TSC tumors (7-10), indicating that TSC-1 and TSC-2 are tumor suppressor gene. TSC-1 and TSC-2 follow the classic retinoblastoma tumor[7,8,9,10] suppressor gene model and appear to function as negative growth regulators[11]. TSC-1 and TSC-2 as tumor suppressor genes when mutated they give rise to abnormal cell proliferation and growth[12,13,14].

The protein product of TSC-2 gene, tuberin, is expressed in variety of different cell types [15,16]. TSC-2 has a 190 kDa protein that contains a Rap1 GTPase-activating protein (GAP)-related domain and a coiled-coil domain believed to mediate its interaction with hamartin (TSC-1) and a carboxyl terminal GTPase[16]. Recently, TSC-2 may function as a tumor suppressor by induction of $p27^{Kip1}$ protein[4]. Interestingly, overexpression of TSC-2 results in reduced cell proliferation *in vitro*[17] and increased amount of cell cycle regulator $p27^{Kip1}$ in rat fibroblast[13]. Also, overexpression of TSC-2 exerts antitumor effect on oral cancer cell line[4]. Therefore, TSC-2 is essential for $p27^{Kip1}$ to regulate the cell cycle because tuberin can retain $p27^{Kip1}$ protein in nuclei of cancer cells[13]. In general, it has been thought that the prognosis of the oral cancer (OSCC) patients who have $p27^{Kip1}$ in the nuclei of their cancer cells should be good[18]. Briefly, TSC-2 may be closely associated with $p27^{Kip1}$ to exert the function as tumor suppressor gene.

In the present study, I conducted to examine that overexpression of TSC-2 gene can suppress the growth of human oral squamous cell carcinoma xenograft by transfecting an expression vector containing sense-oriented rat TSC-2 cDNA.

MATERIALS AND METHODS

All of the research activity was performed in Tokushima University, School of Dentistry, Department of Oral Maxillofacial Surgery and Oncology, Tokushima, Japan, for six months (Oktober 2004 to March 2005).

Cell culture

The original oral squamous cell carcinoma cells were isolated from an oral SCC patient in Tokushima University Dental Hospital, Second Department of Oral Maxillofacial Surgery, Tokushima, Japan[19]. The tumor cells were moderately differentiated SCC of tongue and were not invasive into the muscle layer. The cells were established from a cervical lymph-node metastasis. Cancer cells (B88) were isolated and cloned. The cloned cells were cultured on Petri dishes with the Dullbeco's modified Eagle medium (DMEM: Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS, Moregate BioTech, Bulimba, Australia) and 100 mg/ml streptomycin, 100 units/ml penicillin (Gibco, Grand Island, NY, USA) and incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cancer cells were passaged to new dishes before confluence. In addition, selected clones obtained after transfection were maintained in the same medium supplemented with Geneticin (800 mg/ml G418, Sigma, MO, USA).

Construction of a mammalian expression vector

The mammalian expression vectors pcDNA 3.1-TSC-2 containing sense-oriented rat TSC-2 cDNA was constructed as follows: pcDNA3.1(+) (Invitrogen, Carlsbad, CA) is mammalian expression vectors containing a CMV promoter. PcDNA3.1(+) was digested with Xba 1 (Takara Biomedicals, Kusatsu, Japan) and Xho 1 (Takara Biomedicals) and dephosphorylated by calf intestinal alkaline phosphatase (Roche Diagnostics, Mannheim,

Germany). The rat TSC-2/pBluescript including the rat TSC-2 cDNA fragment (5.4 kbp Xba 1 and Xho 1 fragment) was obtained as a generous gift from Professor Okio Hino (Experimental Pathology, The Cancer Institute, Tokyo, Japanese Foundation for Cancer Research). This fragment was ligated to the prepared cloning site of pcDNA3.1(+) by T4 DNA ligase (Takara Biomedicals). The direction of the ligated fragment was confirmed by sequencing analysis (FIGURE 1)[4].

Transfection of TSC-2 gene

Cells ($5x10^5$ cells/dish) were seeded in 100 mm culture dishes (Falcon) in DMEM supplemented with 10% FCS. Twenty-four hours later, the cells were transfected with 5 mg of pcDNA3.1-TSC-2 or pcDNA3.1 without insert (empty vector or neo) by using the Superfect reagent (Qiagen, Hilden, Germany). The cells were incubated for 48 hours in DMEM containing 10% FCS. Then, trypsinized and seeded at a 1:5 ratio in 100 mm culture dishes in DMEM containing 10% FCS. Forty-eight hours later, the cells were switched to a selective medium containing Geneticin (800 mg/ml G418). After 14 days of culture in the selective medium, ten representative G418-resistant clones were isolated and expanded in a 24-well cluster dish (Falcon).

***In vivo* tumorigenesis assay**

The tumorigenicity of tumor cells was examined in the nude mouse with Balb/cA Jcl-nu genetic background (CLEA Japan, Inc. Tokyo, Japan). The tumor cells ($1x10^6$ cells/mouse) were suspended in 0.1 ml of saline and injected into the subcutaneous tissue of 5-week-old nude mice using a 27-gauge needle. The size of tumors were determined by first measuring length (L) and width (W) and then calculating the volume ($0.4xLxW^2$)

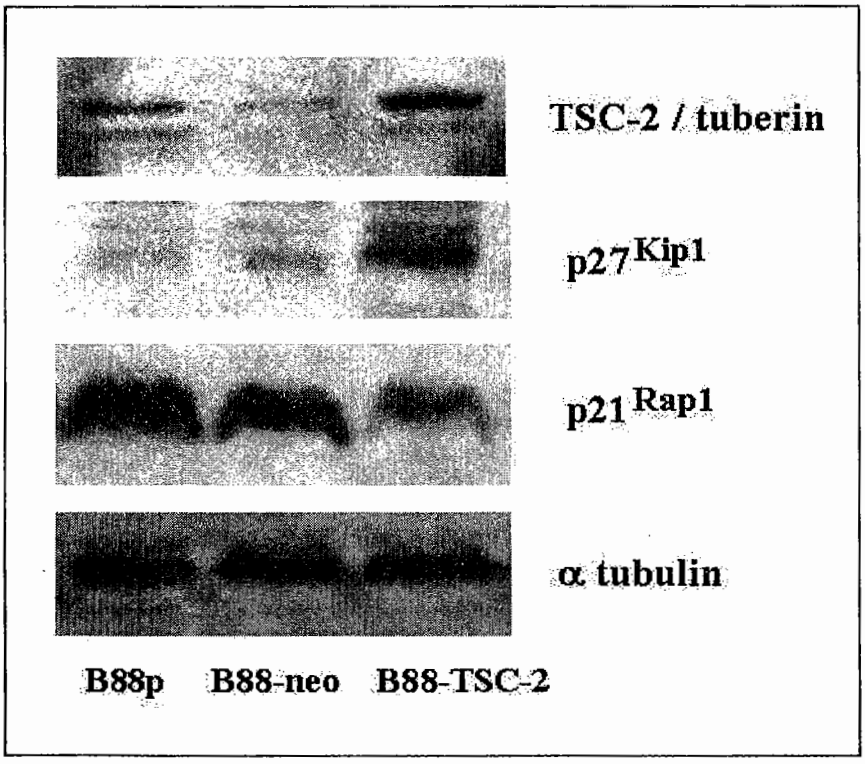

FIGURE 1. Expression of TSC-2 (tuberin) in the transfectants. Cell lysates were prepared from parental cells (B88), control cells (B88-neo) and the sense transfectants (B88-TSC-2).

every 3 days. The body weight of the mice were also measured every 3 days. The mice were sacrificed 24 days after inoculation.

Western blotting

Cell lysates were prepared from the xenograft tumor tissue. Briefly, samples containing equal amounts of protein (50 mg) were electrophoresed on a SDS-polyacrylamide gel and transferred to a nitrocellulose filter (PVDF membrane: Bio Rad, 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 Tuberin (TSC-2) protein (N-19, rabbit polyclonal antibody, SantaCruz Biotechnology, CA, USA), p27 protein (clone 1B4, monoclonal antibody, Novocastra Laboratories, New Castle, UK), Rap1 protein (121, rabbit polyclonal antibody, Santa Cruz Biotechnology, CA, USA) as the primary antibody and an Amersham ECL kit (Amersham Pharmacia Biotech). Anti-a-tubulin monoclonal antibody (Zymed laboratories, San Fransisco, CA, USA) was used for normalization of Western blot analysis.

Statistical analysis

Statistical analysis was performed with a Stat Works Program for Macintosh computers (Cricket Software, Philadelphia, PA, USA). Data were analyzed for statistical significance with One way Anova and Student's *t*-test.

RESULTS

Expression of TSC-2, $p27^{Kip1}$ and Rap1 by Western blotting analysis

After transfection with pcDNA3.1-TSC-2 or pcDNA3.1-neo, I obtained more than 100 G418-resistant colonies in sense transfectants and isolated 10 representative G418-resistant clones in sense transfectants. They were screened for xenograft to the nude mouse. Western blotting analysis revealed that the up-regulation of tuberin and $p27^{Kip1}$ in sense-TSC-2 transfectants compared to that in parental cells or control cells which were transfected with pcDNA3.1 without insert (B88-neo). Contrarilly, the down-regulation of Rap1 appeared in sense-TSC-2 (FIGURE 2).

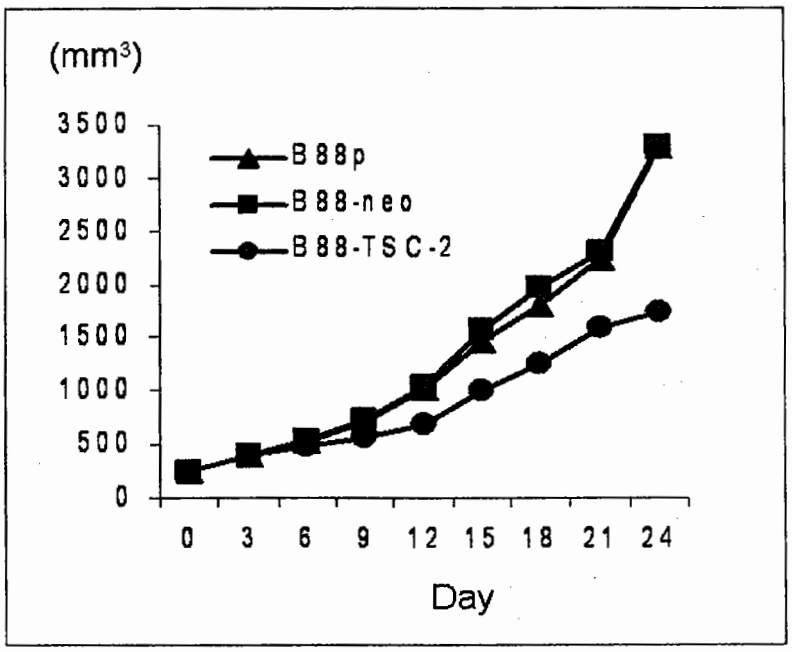

FIGURE 2. Growth of tumors formed by transfectants in nude mice. Tumor cells were suspended in 0.1 ml of serum-free medium and injected into the subcutaneous tissue of nude mice. Each group had 5 mice. The values shown are the mean of five tumors (mm^3); bars, SD, *, $P < 0.01$ compared to that of control cells by One-way Anova.

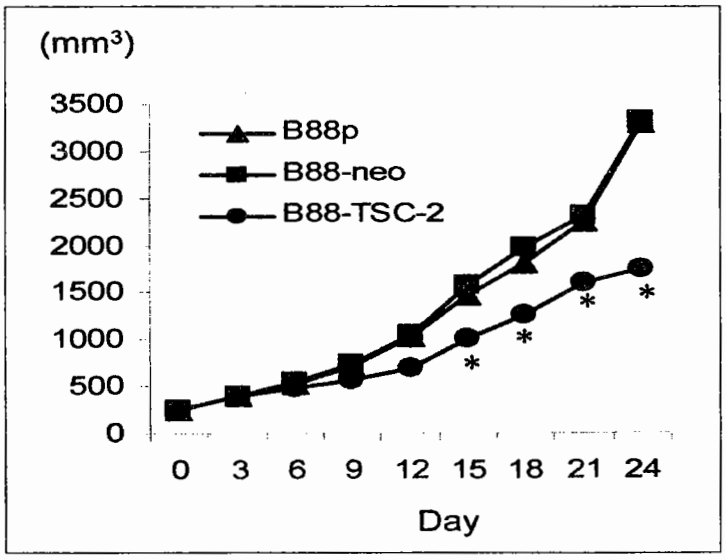

FIGURE 3. Change of body weight in nude mice. The body weight of the mice was measured every 3 days. Each group had 5 mice

***In vivo* tumorigenicity of the transfectanst**

The Result of this experiment showed that all five mice that received parental cells (B88) developed moderate size tumor, and all five mice that received control cells (B88-neo) also developed moderate size tumors. All five mice that received sense transfectants (B88-TSC-2) developed small tumors. As shown in FIGURE 3, tumors induced by TSC-2 up-regulated transfectants become much smaller than those of control cells and parental cells. FIGURE 3 revealed that during the experimental period, no loss of body weight was observed in each group.

DISCUSSION

The autosomal dominant disease tuberous sclerosis complex (TSC) is caused by mutation in either TSC-1 on chromosome 9q34 encoding hamartin, or TSC-2 on chromosome 16p13.3 encoding tuberin. Mutations of either TSC-1 or the TSC-2 gene result in the syndrome of tuberous sclerosis that affects multiple organs with the development of hamartomas including cortical tuber of the brain, angiofibroma of the skin, rhabdomyoma of the heart and angiomyeolipoma of the kidney[20]. TSC-2 seemed to play an important role in exerting the function of $p27^{Kip1}$ protein as a cell cycle regulator by retaining $p27^{Kip1}$ protein in nuclei of cancer cells[13]. However, the true biochemical functions of TSC-2 as a tumor suppressor and the underlying mechanisms responsible for pathogenesis of TSC-related hamartomas have not been clarified yet.

In the present study overexpression of TSC-2 exerted the antitumor effect on human oral cancer cells whether they have high expression of $p27^{Kip1}$ protein. These finding may suggest that TSC-2 can regulate the $p27^{Kip1}$ protein at up-stream of $p27^{Kip1}$. It means that TSC-2 may be more important as a prognostic factor in oral SCC rather than $p27^{Kip1}$. On the contrary, expression of TSC-2 on cells have low expression of Rap1. This finding showed that TSC-2 may function as a tumor suppressor protein by inhibiting Rap1 activity which associated with transformation and tumor formation *in vivo* in some cancer cells[17].

The next results revealed a significant suppression of tumor growth of sense transfectants (B88-TSC-2 cells) *in vivo*. The transfectants did not affect the body weight of the nude mice. These findings may suggest that up-regulation of TSC-2 in tumor cells will be safe for the body and TSC-2 can be used as molecular target for gene therapy if the precise function of TSC-2 can be clarified.

In conclusion, TSC-2 might exert the antitumor effect on oral squamous cell carcinoma through the induction of $p27^{Kip1}$ and inhibition of Rap1 activity.

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