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ANTI TUMOR ACTIVITY OF PCDNA3.1-TUBEROUS a COMPLEX-2 SENSE IN A HUMAN ORAL TONGUE CANCER (SP-C1) CELL LINE

(AKTIVITAS ANTI TUMOR PCDNA3.1-TUBEROUS SCLEROTIC COMPLEX-2 SENSE PADA SEL KANKER LIDAH MANUSIA (SP-C1))

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Abstract

Tuberous sclerosis complex (TSC) is a hamartomatous disease with defects in tuberin (TSC2) that can be autosomal dominant inheritance or spontaneous mutation. Mutation of the TSC-2 gene encoding tuberin on chromosome 16p13.3 increases to the clinical disorder of tuberous sclerosis characterized by the development of hamartomas. In the present study, the antitumor activity of pcDNA3.1-TSC2 sense in an oral tongue cancer *Supri's clone*-1 (SP-C1) was investigated. An expression vector containing sense-oriented rat TSC-2 pcDNA with pcDNA3.1 and transfected to cell (SP-C1) to regulate the expression of TSC-2 gene in each transfectant was constructed. MTT assay was performed to examine the SP-C1 cell growth suppression transfected by pcDNA3.1-TSC2. Caspase-3 and -9 were conducted to observe the induction of cell apoptosis. Western blotting analysis was carried out to determine the protein level of TSC-2, $p27^{Kip1}$ and α-tubulin. The results showed that, overexpression of TSC-2 exerted the growth inhibitory effect of SP-C1 cell and markedly increased apoptosis via caspase-3 and -9 pathways (P=0.001). Sense-oriented SP-C1-cDNA3.1-TSC-2 cancer cells have a high expression of $p27^{Kip1}$. In conclusion, pcDNA3.1-TSC-2 sense increased the antitumor activity of oral tongue cancer SP-C1 cell through $p27^{Kip1}$ induction.

Key words: TSC-2, apoptosis, oral tongue cancer SP-C1, cell growth, $p27^{kip1}$

Abstrak

Tuberous sclerosis complex (TSC) merupakan suatu penyakit hamartoma dengan kelainan pada tuberin (TSC-2) akibat mutasi spontan atau warisan secara autosom dominan. Mutasi gen TSC-2 pada kromosom 16p13.3 meningkatkan kelainan tuberous sclerosis secara klinik dengan karakterisasi berkembangnya hamartoma. Pada penelitian ini dilakukan uji aktivitas antitumor pcDNA3.1-TSC-2 sense pada sel kanker lidah manusia *Supri's clone*-1 (SP-C1). Konstruksi ekspresi vektor yang mengandung sense rat TSC-2 pcDNA dengan pcDNA3.1 ditransfeksikan ke sel SP-C1 untuk meregulasi ekspresi gen TSC-2 pada setiap transfeksi. Uji MTT dilakukan untuk menganalisis hambatan pertumbuhan sel SP-C1 yang ditransfeksi dengan pcDNA3.1-TSC-2. Caspase-3 dan -9 digunakan untuk mengobservasi induksi apoptosis sel. Analisis Western blotting dipakai untuk menentukan tingkat protein TSC-2, $p27^{Kip1}$ dan α-tubulin. Hasil penelitian, peningkatan ekspresi TSC-2 menunjukkan efek hambatan pertumbuhan sel SP-C1 dan peningkatan apoptosis melalui jalur caspase-3 dan -9 (P=0,001). Sel kanker SP-C1-pc DNA 3.1-TSC-2 mempunyai ekspresi protein $p27^{Kip1}$ yang tinggi. Kesimpulan, pc DNA 3.1-TSC-2 sense meningkatkan aktivitas antitumor sel kanker lidah SP-C1 melalui induksi $p27^{Kip1}$.

Kata kunci: TSC-2, apoptosis, kanker lidah, pertumbuhan sel, $p27^{Kip1}$

INTRODUCTION

Cancers of the oral cavity present a major health problem, as indicated by their high incidence in many parts of the world. In Southeast Asia countries, oral cancers are the most common form of cancer and constitute about a third of all cancers.[1] Oral cancers are characterized by a high degree of local invasion and a high rate of metastasis to the cervical lymph nodes. It frequently shows local

recurrence after initial treatment, probably due to micro invasion and/or micro metastasis of the tumor cells at the primary site.[2] Treatment of oral cancer is conventionally a combination of surgery, radiotherapy, and chemotherapy. However, the overall survival rates have not improved significantly in the last two decades.[3] Also, the prognosis has not changed during the past 10 years. [2] This highlights the necessity for continued efforts to improve the treatment modalities using TSC2 gene.

Tuberous sclerotic complex (TSC) is known as 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 and oral.[4,5] Linkage analysis of TSC resulted in the identification of two distinc genetic loci on chromosom 9 and 16.[6] These genes are hamartin/TSC-1 (9q34) and tuberin/TSC-2 (16q13).[7] Germ-line TSC-1 and TSC-2 mutation appear to be inactivating and loss of heterozygosity at either the TSC-1 or TSC-2 region occurs in TSC tumors.[8-9] Indicating that TSC-1 and TSC-2 are tumor suppressor gens. TSC-1 and TSC-2 follow the classic retinoblastoma tumor suppressor gene models and appear to function as negative growth regulators.[10] TSC-1 and TSC-2 as tumor suppressor genes and when mutated, they give rise to abnormal cell proliferation and growth.[11] The protein product of TSC-2 gene, tuberin, is expressed in variety of different cell types.[12] 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.[13] Recently, TSC-2 may function as a tumor suppressor by induction of $p27^{Kip1}$ protein.[5] Interestingly, overexpression of TSC-2 results in reduced cell proliferation *in vitro* [14] and increased amounts of the cell cycle regulator $p27^{Kip1}$ in rat fibroblast.[10] 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.[10] In addition, TSC-2 may be closely associated with $p27^{Kip1}$ to exert the function as tumor suppressor gene.

The purpose of the study was to investigate the antitumor activity of pcDNA3.1-TSC-2 sense in oral tongue cancer cell (SP-C1 cell) through induction of $p27^{Kip1}$.

MATERIALS AND METHODS

SP-C1 cells were isolated from a cervical lymph-node metastasis of an oral squamous cell carcinoma patient in our laboratory.[15] 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.

The mammalian expression vectors pcDNA3.1-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 Dr. Koji Harada (Second Department of Oral Maxillofacial Surgery, Tokushima University, Japan). 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. Furthermore, cells ($5x10^5$ cells/dish) were seeded in 100 mm culture dishes (Falcon, USA) in DMEM supplemented with 10% FCS. Twenty-four hours later, the cells were transfected with 5 g of pcDNA3.1-TSC-2 or pcDNA3.1 without inserted (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 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).

SP-C1-transfected cells were seeded on 96-well plates (Falcon, NJ, USA) at 2×10^4 cells per well in DMEM containing 10% FCS. Cells were incubated for 24 and 48 hours. After incubation, the number of cells was quantitated by BioRad microplate reader machine (Sigma-Aldrich, USA) with 540 nm wavelength. MTT solution contains 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenytetrazolium bromide (Sigma-Aldrich) was used in examination. [17]

Cell lysates were prepared from SP-C1 transfected cells from a Falcon tissue culture. Briefly, samples containing equal amounts of protein (50 μg) 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 37^0C for 1 hour and then incubated with a

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1: 500 dilution of the monoclonal antibody against Tuberin (TSC-2) protein (N-19, rabbit polyclonal antibody, Santa Cruz Biotechnology, CA, USA), and 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.

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-C1 transfected with pcDNA3.1-TSC2 or pcDNA3.1-neo were incubated with the substrate (DVED-pNA or LEHD-pNA) in the assay buffer for 2 hours at 37^0C. Absorbance was measured at 405 nm using a microplate reader (BioRad, USA). Each determination was performed in triplicate.

Statistical analysis was performed with a Stat Works Program for Macintosh computers (Cricket Software, Philadelphia, PA, USA). Data was analyzed by Post Hoc test (Student's *t*-test) with significant level 95%.

RESULTS

Relative cell number was evaluated by comparing the absorbent of each cell using MTT assay on 24 and 48 hours. Cell number of SP-C1-pcDNA3.1-TSC-2 was significantly decreased compared with that of SP-C1-pcDNA3.1-neo (untreated control) on 24 and 48 hours (p =0,001) (Figure 1).

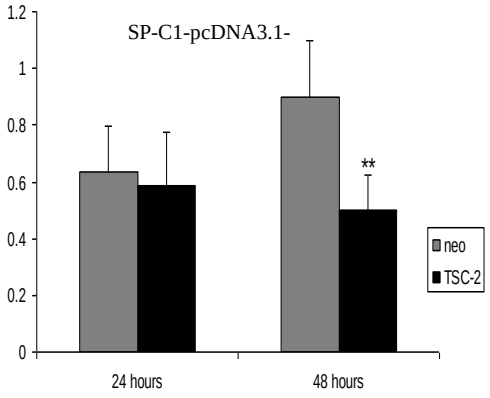

Figure 1. Relative cell number of SP-C1-pcDNA3.1-TSC-2 and SP-C1-pcDNA3.1- neo at 24 and 48 hours by MTT assay (**, p < 0.001)

Western blotting analysis revealed the up-regulation of TSC2 and $p27^{Kip1}$ were detected in sense-TSC-2 transfectans when compared with that in control cells which were transfected with pcDNA3.1-neo. The expression of α-tubulin as an internal control was approximately the same in all of the tumors (Figure 2).

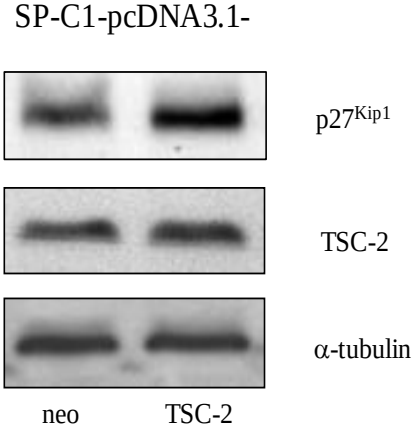

Figure 2. Expressions of TSC-2, $p27^{Kip1}$ and -tubulin in SP-C1-pcDNA3.1-TSC-2 and SP-C1-pcDNA 3.1- neo by Western blotting analyzed

The activity of caspase-3 and -9 in sense-TSC-2 transfectans or control cells was examined using colorimetric assay. As seen in Figure 3, TSC-2 transfectans was markedly increased the proteolytic activities of caspase-3 (2.4 fold) and -9 (1.8 fold) as compared with that of control cells (p=0.000) (Figure 3).

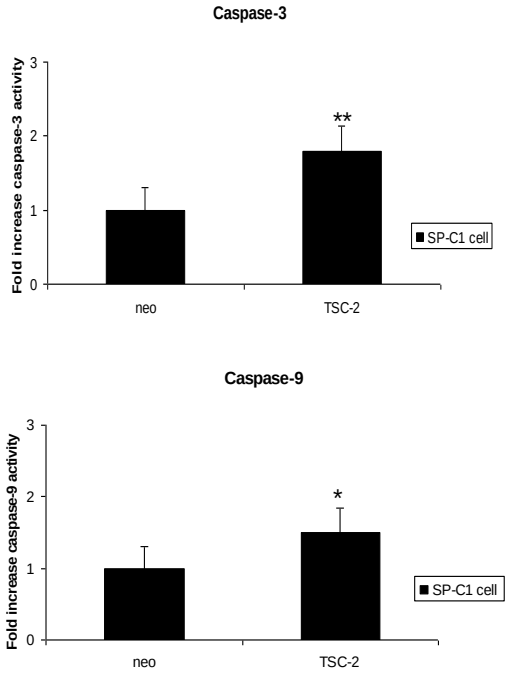

Figure 3. Activation of caspase-3 and -9 in SP-C1-pcDNA3.1-TSC-2 and SP-C1- pcDNA 3.1- neo at 48 hours incubated (**, p < 0.001 and *, p < 0.05)

DISCUSSION

Treatment strategy using gene transfection technique on human head and neck cancer included oral tongue cancer has a main attention of clinicians or oncologists in the world and in the last decade. TSC-2 is used as a tumor suppressor gene and widely used in suppression of TSC-2 syndrome (hamartoma). 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 the 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.[19] 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.[10] 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 tongue 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 $p27^{Kip1}$. Next results revealed that a significant suppression of tumor growth of sense transfectant (SP-C1-TSC-2 cells) *in vitro*. 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. Furthermore, apoptosis induction was detected in SP-C1-pcDNA3.1-TSC2 sense through high level of caspase-3 and-9 compared with that of SP-C1-pcDNA3.1-neo. It is suggested that apoptosis induction of SP-C1 cancer cell can arise via extrinsic and intrinsic pathways. Azuma *et al.* [20] reported that high level of proteolytic activity caspase-3 showed a high cleavage of protein in receptor-induced apoptosis (extrinsic pathway), and induction of caspase-9 level revealed a high proteolytic activity in chemical-induced apoptosis (intrinsic pathway).

In conclusion, TSC-2 may exert the antitumor effect on human oral tongue cancer (SP-C1) cell through $p27^{Kip1}$ protein induction.

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References

1. Petersen PE, Yamamoto T. Improving the oral health of older people: the approach of the WHO Global Oral Health Programme. Community Dent Oral Epidemiol 2005; 33(2): 81-92.
2. Almofti A, Uchida D, Begum NM, Tomizuka Y, Iga H, Yoshida H, Sato M. The clinico-pathological significance of the expression of CXCR4 protein in oral squamous cell carcinoma. Int J Oncol 2004; 25: 65-71
3. Gabriel EM, Jatoi I. Breast cancer chemoprevention. Expert Rev Anticancer Ther 2012; 12(2): 223-8.
4. Ibáñez Micó S, Domingo Jiménez R, Guillén Navarro E, Casas Fernández C. Tuberous sclerotic complex is there evidence to establish a genotype-phenotype correlation?. A Pediatr (Barc) 2011; 74(6): 421-3.
5. Kawaguchi S, Harada K, Supriatno, Yoshida H, Sato M. Overexpression of tuberous sclerosis complex 2 exert antitumor effect on oral cancer cell lines. Oral Oncol 2003; 39: 836-41.
6. Knowles MA, Habuchi T, Kennedy W, Cuthbert-Heavens D. Mutation spectrum of the 9q34 tuberous sclerotis gene TSC1 in transitional cell carcinoma of the bladder. Cancer Res 2003; 63(22): 7652-6.
7. Leung AK, Robson WL. Tuberous sclerotic complec: a review. J Pediatr Health Care. 2007; 21(2):108-14.
8. Nelson M, Quinonez S, Ackley T, Iyer RK, Innis JW. Multiple congenital anomalies and development delay in a boy associated with a de novo 16p13.3 deletion. Am J Med Genet 2011; 155A(3): 612-7.
9. Mizuno S, Fukushi D, Kimura R, Yamada K, Yamada Y, Kumagai T, Wakamatsu N. Clinical and genomic characterization of sibling with a distal duplication of chromosome 9q (9q34 1q-ter). Am J Med Genet A 2011; 155A(9): 2274-80.
10. Uhlmann EJ, Apicelli AJ, Baidwin RL, Burke SP, Bajenaru ML, Onda H. Heterozygosity for the tuberous sclerosis complex (TSC) gene products results in increased astrocyte number and decreased p27-Kip1 expression in TSC+/- cells. Oncogene 2002; 21: 4050-9.
11. Hengstchlager M, Rodman DM, Milolozoa A, Hengstchlager-Ottnad E, Rosner M, Kubista M. Tuberous sclerosis gene products in proliferation control. Mutat Res 2001; 488: 233-9.
12. Milolozoa A, Rosner M, Nellist M, Halley D, Bernaschek G, Hengtschlager M. The TSC-1 gene

product, hamartin, negatively regulates cell proliferation. Hum Mol Genet 2000; 9: 1721-7.
13. Han JM, Sahin M. TSC1/TSC-2 signaling in central nervous system. FEBS Lett. 2011; 585(7): 973-80.
14. Khare L, Astrinidis A, Senapedis W, Adams PD, Henske EP. Expression of wild type and mutant TSC-2, but not TSC-1, causes in increase in the G1 fraction of the cell cycle in HEK293 cells. J Med Genet 2002; 39(9): 676-80.
15. Supriatno, Harada K, Yoshida H, Sato M. Basic investigation on the development of molecular targeting therapy against cyclin-dependent kinase inhibitor p27Kip1 in head and neck cancer cells. Int J Oncol 2005: 27: 627-35.
16. Lau N, Uhlmann EJ, Von Lintig FC, Nagy A, Boss GR, Gutmann DH, Guha A. Rap1 activity is elevated in malignant astrocytomas independent of tuberous sclerosis complex-2 gene expression. Int J Oncol. 2003; 22(1): 195-200.
17. Harada K, Supriatno, Yoshida H, Sato M. Low p27Kip1 expression is associated with poor prognosis in oral squamous cell carcinoma. Anticancer Res 2002; 22(5): 2985-9.
18. Supriatno, Harada K, Hoque MO, Bando T, Yoshida H, Sato M. Overexpression of $p27^{Kip1}$ induces growth arrest and apoptosis in an oral cancer cell line. Oral Oncol 2002; 38: 730-6.
19. Schönberger A, Gembe E, Grote A, Witt JA, Elger CE, Bien CG, Urbach H, Becker AJ, Niehusmann P. Genetic analysis of tuberous-sclerotis gene 1 and 2 in nonlesional focal epilepsy. Epilepsy Behav 2011; 21(3): 233-7.
20. Azuma M, Harada K, Supriatno, Tamatani T, Motegi K, Ashida Y and Sato M. Potentiation of induction of apoptosis by sequential treatment with cisplatin followed by 5-fluorouracil in human oral cancer cells. Int J Oncol 2004; 24: 1449-55.

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