

Characteristics of New Primary Oral Cancer (Sp-C1) Cell and Antitumor Activity of Oligonucleotide p27Kip1 Sense on a Sp-C1 Cell

Supriatno¹, Sartari Entin Yuletnawati^{2,3}

¹Department of Oral Medicine, Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta, Indonesia

²Post-graduate student, Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta, Indonesia

³Faculty of Dentistry, Muhammadiyah University, Surakarta, Indonesia

Abstract: *New primary oral cancer (Sp-C1) cell has some characteristics of cell growth, expression of anti-apoptosis protein and positive regulatory protein of cell cycle. Characteristics of Sp-C1 cell is very important to facilitate the researchers using an in vitro model of oral cancer cells. In the present study, p27Kip1 sense (p27 S), p27Kip1 antisense (p27AS) and p27Kip1 neo (p27 neo) were transfected into Sp-C1 cells. The aim of study was to introduce the new primary oral cancer cell with their characteristics and to examine the antitumor activity of oligonucleotide p27 S on Sp-C1 cell in vitro. Isolation of Sp-C1 cell was derived from cervical lymph node tissue of tongue cancer patient. As a final step of isolation, new fibroblast and primary oral cancer cells were cultured and stocked. The efficiency of p27 S and p27 AS transfection in cell growth inhibition test was confirmed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Colorimetric assay caspase-3 and caspase-9 were carried-out to know the apoptosis induction. The efficiency of oligonucleotide p27 transfection at protein level was confirmed by Western blotting analysis. Results of study revealed characteristics of Sp-C1 was relative fast in cell growth and positive expression of Skp2, α -tubulin, CDK-2, cyclin-E, and maspin protein. Furthermore, Sp-C1 cell growth was markedly suppressed by p27 S. Also, p27 S-treated cell induced apoptosis characterized by an increase proteolytic activities of caspase-3 and -9. Up-regulation of p27Kip1 protein was detected in Sp-C1 p27 S. In conclusion, Sp-C1 cells had a certain characteristics and appropriate for an in vitro study model of oral cancer. p27 S had a strong antitumor activity on Sp-C1 cell. Targeting this molecule could represent a promising new therapeutic approach for this type of cancer.*

Keywords: cell characteristic, Sp-C1 cell, p27 S, antitumor activity.

1. Introduction

Oral tongue squamous cell carcinoma (OTSCC) is a disease with disruption characteristic or failure of regulatory mechanisms of a multicellular organism multiplication resulting in a change of behavior uncontrolled cell in the oral cavity.¹ OTSCC is caused by the destruction of the basic regulatory mechanisms of cells behavior, particularly mechanisms of cell growth and differentiation. OTSCC is characterized by DNA damage and autonomous cell growth that do not respond to normal growth regulation.² It has a high mortality rate and it occupies the 6th position from all types of human cancer. OTSCC is a malignant cancer that has the lowest average length of life among the major malignant cancer.³ It has 2-5% evidenced of all type human cancers. OSCC presents a major health problem, as indicated by their high incidence in many parts of the world.⁴ In Southeast Asia countries, oral cancers are the most common form of cancer and constitute about a third of all cancers.³ Oral cancer is 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.⁴ Interestingly, the overall survival rates have not improved significantly in the last two decades and the prognosis has not changed during the past 10 years.⁵ This highlights the necessity for continued efforts to improve the research and treatment modalities.

Sp-C1 cell is a new primary oral cancer cell derived from cervical lymph nodes epithelial tissue of tongue cancer patient with mild differentiation and not involving the muscles. Sp-C1 cells have been used for some in vitro research model to examine the effectiveness and efficiency of synthetic drugs, herbal medicine and gene therapy.⁶⁻⁹ In the present study, we introduced a new primary oral tongue cancer cells (Sp-C1 cell) with their characteristics and examined the antitumor activity of oligonucleotide p27 S, p27 AS and p27 neo on Sp-C1 cell.

p27Kip1 is a universal cyclin-dependent kinase inhibitor that directly inhibits the enzymatic activity of cyclin-CDK complexes, resulting in cell cycle arrest at G₁.¹⁰ p27Kip1 is an important prognostic factor in various types of malignancies. Recently, decreased expression of p27Kip1 has been frequently detected in human cancer.¹¹⁻¹³ In addition, loss of p27Kip1 has been associated with disease progression and an unfavorable outcome in several types of malignancies.¹⁴ Furthermore, mice lacking the p27^{Kip1} gene show an increase in body weight, thymic hypertrophy and hyperplasia of pituitary intermediate lobe adrenocorticotrophic hormone cells, adrenal glands and gonadal organ.²¹

2. Materials and Methods

Isolation of new primary cell

New primary cells (Sp-C1 cells) was derived from cervical lymph node tissue of oral tongue cancer cell patient. The tissue has a well differentiation and has not involved muscle

tissue. Fresh tissue was put in petry dish ($\phi = 60$ mm) contained sterile phosphate buffered saline (PBS, Sigma-Aldrich, USA) and penicillin-streptomycin (Penstrep; Gibco, USA) solution. In the sterile laminar-flow clean bench (Thermo Scientific, USA), fresh tissue was cut in small pieces using sterile scissors or scalpel no 11 and incubated for 24 hours at a temperature of 37°C . New cells and fibroblast as result of cutting tissue were checked under a light microscope (Olympus, Japan) with 100x magnification. The primary oral cancer cells were marked under petry dish and separated from fibroblast cells using serial EDTA-trypsin procedure.

Isolation of fibroblasts cells was carried-out with Whatman paper dipped in a solution of 0.25% Trypsin-EDTA (Sigma-Aldrich, USA) in a petri dish (Falcon, USA). Fibroblast cells were cultured in Dulbecco's Modified Eagle Media (DMEM, Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS, Moregate, Australia). Fibroblasts were incubated at a temperature of 37°C and CO_2 5%. Then, a new fibroblast cells were stocked and stored in the refrigerator at a temperature of -80°C .

Isolation of a new oral cancer cells were done by the same procedure, namely Whatmann paper soaked in a solution of 0.25% Trypsin-EDTA (Sigma-Aldrich, USA) in a petry dish. The paper was placed in each new cell according to mark or label at under the dish and incubated at 37°C for 5 minutes. Each new cell in the paper was placed in one petry dish (labeled clone-1 to -10). Then, papers were transferred to a new petry dish containing DMEM 10% FBS and incubated for 24 hours. Each dish of new primary cells was observed every day under a light microscope (Olympus, Japan) with 100x magnification. In addition, the new primary cell was ensured free of fibroblasts. Cells with the fastest growth and healthy cells were preserved, cultured and stocked. Finally, a new oral cancer cells was named Sp-C1.

Characteristic of Sp-C1 cells

Characteristics of Sp-C1 cells were determined by the rapid cell growth, cell morphology, cell adhesion, tumor protein expression was examined by Western blotting analysis and in vivo tumorigenesis in animal models. This research was done since 2004 to 2013.

Antisense experiments

Antisense experiments were performed as described previously.¹³ Two oligonucleotides for p27 (Fasmac Co., Kanagawa, Japan) were synthesized as follows: S, 5'-GGCGCAGGAGAGCCA-3' and AS, 5'-TGGCTCTCCTGCGCC-3' (the AS direction of human Kip1 cDNA nucleotide, 15 mer). The oligonucleotides were delivered into Sp-C1 cell directly according to the manufacturer's instructions.

Cell growth inhibition (MTT assay)

Sp-C1 cells were seeded on 96-well plates (Falcon, USA) at 2×10^4 cells per well in DMEM (Sigma-Aldrich, USA) containing 10% FBS (Moregate, Australia), the day before treatment. Cell was treated with oligonucleotides at final concentration 100 μM . After 24 and 48 hours, the number of cells was quantitated by an assay in which MTT; 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (Sigma-Aldrich, USA) was used.

Activity of caspase-3 and -9

Caspase-3 and -9 activities were measured using the colorimetric assay kit according to the manufacturer's directions. This test is based on the addition of a caspase-specific peptide conjugated to a color reporter molecule p-nitroanilide (p-NA). The cleavage of the peptide by caspase releases the chromophore p-NA, which is quantitated spectrophotometrically at 405 nm. Briefly, equal amounts of cell extracts prepared from Sp-C1 cell transfected with p27Kip1 S, AS and UC were incubated with the substrate (DVED-pNA and LEHD-pNA; BioVision colorimetric assay kit, CA, USA) in the assay buffer for 2h at 37°C . Absorbance was measured at 405 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Each determination was conducted in triplicate.¹⁵

Western Blotting Analysis

Cell was treated with oligonucleotides for 48 hours. Cell lysates were prepared from the treatments as follows: Sp-C1 p27Kip1 S, Sp-C1 p27Kip1 S and Sp-C1 p27Kip1 UC washed with 100 mM phosphate-buffered saline (PBS) and lysed with 50 mM N-2-hydroxyethyl piperazine-N-2-ethanesulfonic acid, HEPES (pH 7.5) containing 150 mM NaCl, 1% Triton X-100, 1.5 mM MgCl_2 , 1 mM EDTA, 10 mM sodium pyrophosphate, 100 mM sodium orthovanadate, 100 mM NaF, 100 mM p-nitrophenyl phosphate, 5 U/ml aprotinin and 1 mM phenyl-methylsulfonyl fluoride. The protein concentration of the samples was determined by Bio-Rad protein assay. Fifty micrograms of protein samples were electrophoresed on SDS-polyacrylamide gel. Proteins from gels were transferred to PVDF membrane. The membrane was incubated with a 1:1,000 dilution of the rabbit polyclonal antibody or monoclonal antibody against p27 (Clone 1B4), Skp2, CDK-2, cyclin E, MTA-1 and Maspin as the primary antibody and an Amersham ECL kit. Also, anti α -tubulin monoclonal antibody was used for normalization of Western blot analysis.⁴

Statistical analysis

Statistical differences between the means for the different groups were evaluated with Stat View 4.5 (Abacus Concepts, Berkeley, CA) using two-way ANOVA and *t-test*. The significance level was set at 5% for each analysis.

3. Results

Characteristics of Sp-C1 cells

Sp-C1 cell was growing rapidly in DMEM (Sigma-Aldrich, USA) 10% FBS (Moregate, Australia), but it will grow slowly in ROSSWELL Park Memorial Institute (RPMI) 1640 (Sigma-Aldrich, USA) media in cell culture. The specific morphological of Sp-C1 cells is appeared irregular form such as spherical oval, round, oval and flat. Sp-C1 viability cells are always firmly attached at the bottom of the petry base (Falcon, USA) and not floating on the surface of the media, except for death cells. Sp-C1 cells had a positive regulation to anti-apoptosis protein expression maspin and MTA-1, to cell cycle protein (cyclin-dependent kinase 2

(CDK-2) and cyclin-E), to tumor promoter protein (p45Skp2), and to tumor suppressor protein (p27Kip1). α -tubulin was also expressed in Sp-C1 cells (Figure 1).

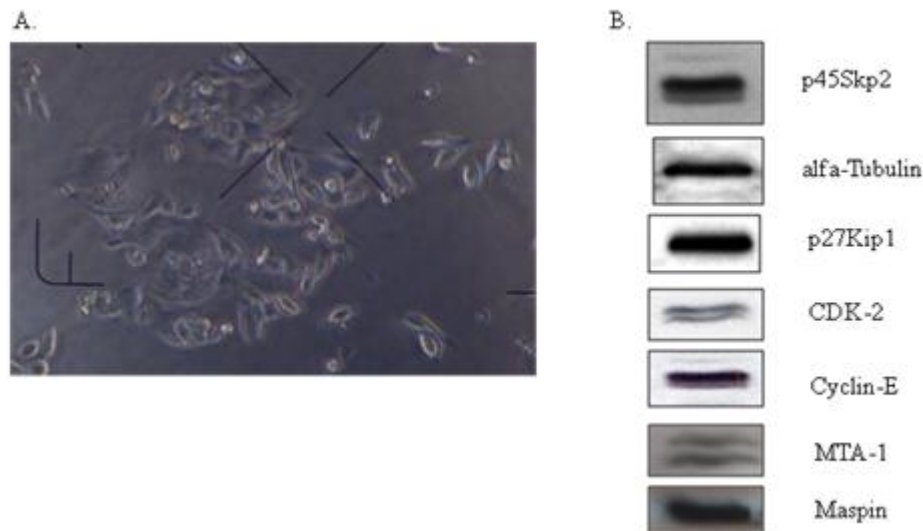


Figure 1: Characteristics of new primary oral cancer (Sp-C1) cells. A. Sp-C1 Cell morphological and growth. B. Proteins expression of Sp-C1 cells analyzed by Western blotting.

In in vivo study models, Sp-C1 cells had a positive tumorigenicity when inoculated at backs of nude mice, but not of Wistar and Sprague Dawley mice (data is not shown).

Cell growth inhibition (MTT assay)

The transfected cells were examined by the MTT assay. Relative cell number was evaluated by comparing the absorbance in each cell. As seen in Figure 2, the cell growth of Sp-C1 cells transfected with oligonucleotide p27Kip1 S was significantly suppressed as compared to that of the p27Kip1 AS and p27Kip1 UC at 24 hours ($P = 0.0380$) and 48 hours ($P = 0.000$).

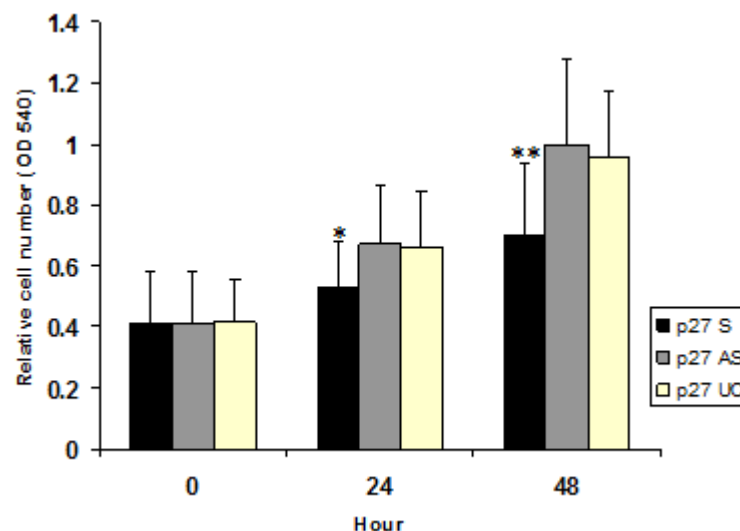


Figure 2: Relative cell number was evaluated by comparing the absorbance in each cell at 24 and 48 hours using MTT assay. Values shown are the mean of six determinations; error bars indicate standard deviations. * $P < 0.05$, ** $P < 0.01$.

Proteolytic activities of caspase-3 and -9

The activity of caspase-3 and -9 in p27Kip1 S, p27Kip1 AS and p27Kip1 UC were investigated. p27Kip1 S showed increased caspase-3 and -9 proteolytic activities as compared

with that of p27Kip1 AS and p27Kip1 UC (Figure 3). Proteolytic activities of caspase-3 in p27Kip1 S was 3.7 fold increased compared with UC. Furthermore, proteolytic activities of caspase-9 in p27Kip1 S was 1.8 fold increased ($P = 0.001$).

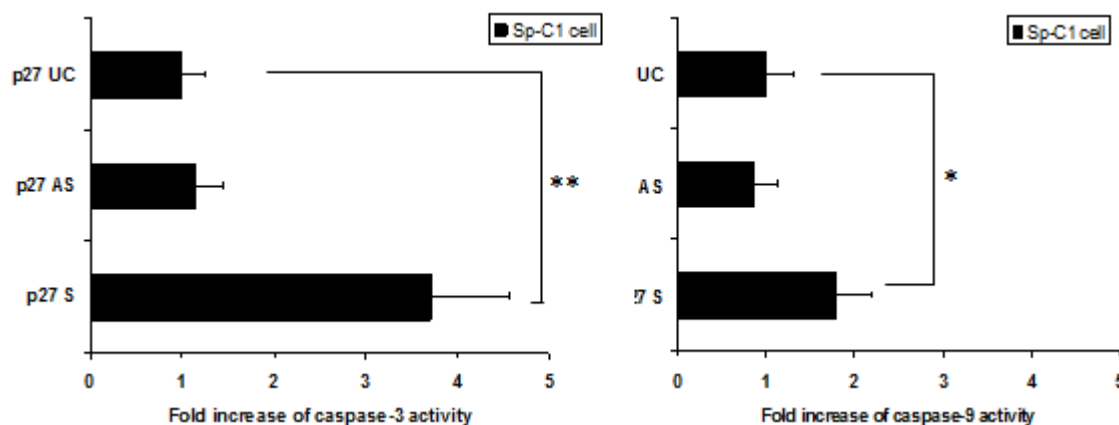


Figure 3: The activity of caspase-3 and -9 in Sp-C1 cells. Cells were transfected with p27 S, p27 AS or p27 UC oligonucleotides. ** Values are shown as mean; error bars indicate standard deviation.

Expression of p27Kip1 protein

To evaluate the efficiency of transfection of oligonucleotide p27 S, AS and UC, the expression of p27Kip1 protein by Western blotting was evaluated. As shown in Figure 4, up-regulated of p27Kip1 protein in Sp-C1 p27 S was detected when compared with that in Sp-C1 p27 UC. The expression of α -tubulin as an internal control was approximately the same in all of the tumors.

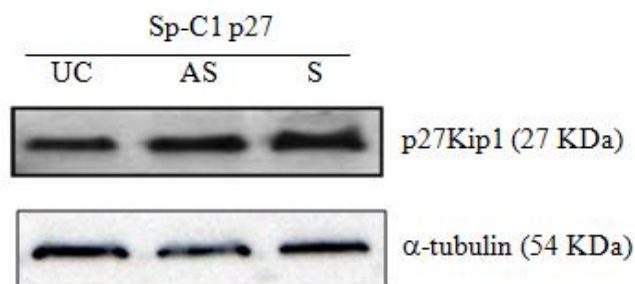


Figure 4: Expression of p27Kip1 and α -tubulin protein determined by western blotting. The Sp-C1 cells was treated with 100 μ M p27 S, AS, or UC for 48 hours.

4. Discussion

Sp-C1 cell cultured in DMEM 10% FBS had a higher growth rate than in RPMI 1640 10% FBS. This condition is one of the characteristics of Sp-C1 cells. Specific features including tumorigenicity which can be either positive or negative depending on the type of mice used in the study. Positive tumorigenicity was seen in nude mice with a Balb/c background, whereas in Wistar and Sprague-Dawley mice injected Sp-C1 cells were not grown (data is not shown). This possibility caused both (Wistar and Sprague-Dawley) are having the good immune system, so that the cells were injected at backs can be lysed.¹⁶ Another characteristics of Sp-C1 cells were anti-apoptotic, tumor promoter and positive regulation of cell cycle proteins expression. Anti-apoptotic proteins or inhibitory apoptosis protein (IAP) such as maspin and MTA-1 protein were expressed in Sp-C1 cells. Similarly, CDK-2 and cyclin E protein that rule as a positive regulator of cell cycle were expressed in Sp-C1 cells through Western blotting analysis. Furthermore, tumor promoter protein likes p45Skp2 was also expressed in that

cells. These indicated that all expressed proteins were triggers tumor protein of cancer cells.

High expression of p45Skp2 indicated Sp-C1 is an oral cancer cells with high cell growth and metastasis aggressiveness. It was reported that high expression of p45Skp2 showed a poor prognosis and a high degree of malignancy in patients with colon cancer¹⁷, lymphoma¹⁸, gastric¹⁹, and lungs²⁰. p45Skp2 knockout (- / -) in nude mice revealed slower growth of cancer cells and increased apoptotic activity compared with that of control or p45Skp2 (+ / +)²¹.

The strategy of therapy with p27Kip1 sense in human head and neck cancer includes human oral tongue cancer becomes the focus of attention in this decade. Sense oligonucleotides p27Kip1 contained phosphorothioate backbone was used to knock down protein expression by inhibiting the translation of the mRNA of a desired target gene¹⁵. Sense and antisense oligonucleotides are synthesized in the hope that they can be used as therapeutic agents-blocking disease processes by blocking the synthesis of a particular protein. This would be achieved by the binding of the sense and antisense oligonucleotide to the mRNA from which that protein is normally synthesized. Binding of the two may physically block the ability of ribosome to move along the messenger RNA or simply hasten the rate at which the mRNA is degraded within the cytosol²². In order to be useful in human therapy, sense and antisense oligonucleotides must be able to enter the target cells; avoid digestion by nucleases, and not cause dangerous side-effects. To achieve these goals, sense and antisense oligonucleotides are generally chemically modified to resist digestion by nucleases, attached to a targeting device such as the ligand for the type of receptors found on desired target cells, antigen receptors directed against molecules on the surface of the desired target cells.¹⁵

In the present study, a sense oligonucleotide strategy to investigate the antitumor activity of p27Kip1 on an oral cancer (Sp-C1) cell that was overexpressing this gene was employed. Transfection of a sense oligonucleotide p27Kip1 into Sp-C1 cells induced strong cell growth suppression followed by induction of apoptosis characterized with increase of caspase-3 and caspase-9 proteolytic activities. An increase in activation of caspase-3 in S-treated cells

strongly suggested that apoptosis had occurred in those cultures. In fact, apoptosis was originally described as a mechanism of controlled or physiological cell death. It is associated with the regulation of cellular homeostasis in organs and the elimination of damaged cells or of cells with deleterious reactivities from the host. Apoptosis is very common in organs with high proliferation activity and in tissue with intense hematopoietic activity. Additionally, apoptosis has been implicated in the progression of a number of pathological conditions, including cancer and autoimmune diseases.²³ As expected from its stronger growth inhibition and apoptosis induction, up-regulation of p27Kip1 protein was detected in Sp-C1 p27⁺ S. It was reported that p27Kip1 sense and p45Skp2 AS induce apoptosis in human oral tongue cancer cells through induction of p27^{Kip1} protein. Since components of apoptotic programs represent promising targets for anticancer therapy, up-regulation of p27Kip1 by the sense oligonucleotide approach could be a useful apoptosis-modulating strategy for treatment of human oral cancers.

In conclusion, Sp-C1 cells are a new primary cell of oral cancer with specific characteristics and suitable for in vitro research model. Sense oligonucleotide p27Kip1 had a high antitumor activity in oral cancer (Sp-C1) cells, targeting this molecule could represent a promising new therapeutics approach for this type of cancer.

5. Acknowledgments

The author heartfully thanks and appreciates to Mrs. Rumbi, Department of Paracytology, Faculty of Medicine, and Mrs. Yuli, LPPT-Universitas Gadjah Mada, Yogyakarta, Indonesia. Thank to all the staff members at the Department of Oral Medicine, Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta, Indonesia.

6. Conflict of Interest

The author wish to express that she has no conflict of interest.

References

- [1] Hook SS, Lin JJ, Dutta A. Mechanisms to control re-replication and implications for cancer. *Curr Op Cell Biol* 2007;19(6):663-71
- [2] Wasil LR, Wei L, Chang C, Lan L, Shair KH. Regulation of DNA Damage Signaling and Cell Death Responses by Epstein-Barr Virus Latent Membrane Protein 1 (LMP1) and LMP2A in Nasopharyngeal Carcinoma Cells. *J virol* 2015; 89(15):7612-24.
- [3] Petersen PE, Yamamoto T. 2005. Improving the oral health of older people: the approach of the WHO Global Oral Health Programme. *Comm Dent Oral Epidemiol* 33(2):81-92.
- [4] Supriatno, Yuletnawati SE, Widiasto A. 2011. Effect of intratumoral injection of mutant type p27Kip1 followed by in vivo electroporation on radiotherapy-resistant human oral tongue cancer xenografts. *Mol Med Report* 4(1): 41-6.
- [5] Almofiti 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.
- [6] Supriatno. Cell cycle arrest analysis of Sp-C1 oral cancer cells induced by biscoclairine alkaloid cepharanthine using flow cytometry FACSscan. *Indon J Cancer* 2010, 3:7-13.
- [7] Supriatno. Docetaxel hydrate inhibits proliferation and metastasis of Sp-C1 oral cancer cell through induction of maspin expression. *Indon J Dent* 2008, 15(1):2-7.
- [8] Achmad H, Satari MH, Oewen R, Supriatno. Antitumor activity of celecoxib agent on the invasion of Sp-C1 oral cancer cells. *J Dent Padjadjaran* 2011, 23(1): 1-5.
- [9] Setiawan AS, Oewen RR, Supriatno, Soewondo W, Sidik, Supratman U. 8-hydroxycudraxanthone G suppresses IL-8 production in SP-C1 tongue cancer cells. *Nat Prod Com (NPC)* 2014, 9(1): 75-8.
- [10] Prasad SB, Yadav SS, Das M, Modi A, Kumari S, Pandey LK, *et al.* PI3K/AKT pathways-mediated regulation of p27(Kip1) is associated with cell cycle arrest and apoptosis in cervical cancer. *Oncol Rep* 2015, 34(2):995-1002.
- [11] Supriatno: Antitumor activity of Papua's Myrmecodia pendans in human oral tongue squamous cell carcinoma cell line through induction of cyclin-dependent kinase inhibitor p27Kip1 and suppression of cyclin E. *J Cancer Res Ther* 2014, 2(3): 48-53.
- [12] Alkarain A, Jordan R and Slingerland J: p27 deregulation in breast cancer: prognostic significance and implications for therapy. *J Mamary Gland Biol Neoplasma* 2004, 9(1):67-80.
- [13] Sion-Vardy N, Freedman J, Lazarov I, Bolotin A and Aviad S: P27Kip1 expression in non-small cell lung cancer is not an independent prognostic factor. *Anticancer Res* 2010, 30(9): 3699-704.
- [14] Chen J, Amos CJ, Merriman KW, Wei Q, Sen S, Killary AM and Frazier ML: Genetic variants of p21 and p27 and pancreatic cancer risk in non-hispanic whites: a case-control study. *Pancreas* 2010, 37(1): 1-4.
- [15] Supriatno. S-phase kinase associated protein-2 antisense induces the growth inhibition and apoptosis in a parotid gland cancer cell. *HK Dent J* 2008, 5:27-30.
- [16] Supriatno, Harada K, Yoshida H, Sato M. Basic investigation on the development of molecular targeting therapy against cyclin-dependent kinase inhibitor p27^{Kip1} in head and neck cancer cells. *Int J Oncol* 2005, 27: 627-635.
- [17] Hershko D, Bornstein G, Ben-Izhak O, Carrano A, Pagano M, Krausz MM, *et al.* Inverse relation between levels of p27Kip1 and of its ubiquitin ligase subunit Skp2 in colorectal carcinoma. *Cancer* 2001, 91:1745-51.
- [18] Chiarle R, Fan Y, Piva R, Boggino H, Skolnik J, Novero D. S-phase kinase-associated protein 2 expression in non-Hodgkin's lymphoma inversely correlates with p27 expression and defines cells in S phase. *Am J Pathol* 2002, 160:1457-66.
- [19] Masuda TA, Inoue H, Sonoda H, Mine S, Yoshikawa Y, Nakayama K, *et al.* Clinical and biological significance of S-phase kinase-associated protein 2 (Skp2) gene expression in gastric carcinoma: modulation of malignant phenotype by Skp2 overexpression, possibly via p27 proteolysis. *Cancer Res* 2002, 62:3819-25.
- [20] Yokoi S, Yasui K, Lizasa T, Takahashi T, Fujisawa T,

- Inazawa J. Down-regulation of SKP2 induces apoptosis in lung-cancer cells. *Cancer Sci* 2003, 94:344-9.
- [21] Nakayama K, Nagahama H, Minamishima YA, Matsumoto M, Nakamichi I, Kitagawa K. Targeted disruption of Skp2 results in accumulation of cyclin E and p27^{Kip1}, polyploidy and centrosome overduplication. *EMBO J* 2000, 19: 2069-81.
- [22] Weiss B, Davidkova G, Zhou LW. Antisense RNA gene therapy for studying and modulating biological processes. *Cell Mol Life Sci* 1999, 55:334-358.
- [23] Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science* 1995, 267: 1456-62.