Expression of Non-Metastatic Protein-23 And Metastatic Associated Protein-1 as a Molecular Target Therapy of an Oral Malignant Burkitt’s Lymphoma Induced by Oligonucleotide P27 Sense

Supriatno¹*, Ana Medawati², Sartari Entin Yulet Nawati³

1. Department of Oral Medicine, Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta, Indonesia.
2. Staff at Dental Study Program, Muhammadiyah University of Yogyakarta, Indonesia.
3. Staff at Soelastri Dental Hospital, Muhammadiyah University of Surakarta, Indonesia.

Abstract
Burkitt’s lymphoma (BL) is an uncommon type of Non-Hodgkin lymphoma (NHL). It is a highly aggressive type of B-cell lymphoma and the most common childhood cancer. Treatment for this cancer is still limited. However, a new strategy for refractory tumor, gene therapy is watched with keen interest.

The aim of study was to investigate the expression of non-metastatic 23 (nm-23) and metastatic associated protein-1 (MTA-1) of an oral malignant Burkitt’s lymphoma induced by oligonucleotide p27 sense (p27 S). The true experimental study with post-test only control group design was confirmed in this study. For detection of nm-23, MTA-1, p27Kip-1 and β- tubulin protein expressions were carried out by Western blotting analysis. Induction of apoptosis was analyzed by double staining using acridine orange-ethidium bromide (AO-EB).

Results revealed the expression of nm-23 and p27Kip-1 protein was markedly increased in cell transfected with p27 sense. Contrarily, down regulation of MTA-1 protein was detected in these cell transfectant. Apoptosis induction was significantly elevated in Raji-p27 S compared with that of p27 AS.

In conclusion, oligonucleotide p27 S has a strong antitumor activity in oral malignant Burkitt’s lymphoma through increasing the apoptosis activity, expression of nm-23 and p27Kip-1 protein induction, and suppressing the expression of MTA-1 protein targeting this molecule could represent a promising new therapeutic approach for this type of cancer.

Keywords: Burkitt’s lymphoma cell, nm-23, MTA-1, p27 sense, Western blotting.

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Introduction
Burkitt’s lymphoma (BL) is one of the most aggressive malignancies of lymphoid origins and accounts for 3-5% of all lymphomas. BL is a high grade B-cell neoplasm. Usually found in the pediatric population, BL represents 40% of childhood NHL.¹ The highest incidence is found in the endemic form of equatorial regions of Africa and Papua-New Guinea where it accounts for 50-70% of all pediatric malignancies.²

¹Corresponding author:
Dr. Supriatno., DDS., M.Health., MDSc., Ph.D
Assoc. Professor at Department of Oral Medicine, Faculty of Dentistry,
Universitas Gadjah Mada, Yogyakarta, Indonesia
E-mail: supriatno_fkg@ugm.ac.id

Development of BL cancer is a complex process consisting of transformation, tumor growth, invasion and metastasis. Invasion and metastasis of cancer cells is an integral component of cancer growth and development. Cell motility and invasion are tightly regulated by growth factors, cytokines, and extracellular matrix components in the micro environment of cancer cells.³⁻⁴ Invasion, metastasis and therapeutic resistance are important phenomenon in the malignancy and progression of cancer cells. The abnormality of cell cycle regulation and the imbalance between dead cells and living cells is one of the triggers for the occurrence of malignancy and aggressiveness of cancer cells.⁵ Aggressiveness and biological activity of cells can be identified and observed through specific biomarkers for invasion and metastasis activity. Non-metastatic-23 (nm-23) and metastatic associated protein-1 (MTA-1) expressions are
one of the main biomarkers in inhibiting and/or triggering the invasion and metastasis activity of human cancer cells.  

The nm-23 protein is a metastatic inhibitory protein that has been shown to have an inverse correlation with the potential for metastases in several types of human cancer cells. Decreased expression of nm-23 is significantly associated with aggressive behavior in melanoma, breast, colon, and gastric carcinoma. Conversely, a high increase in nm-23 expression is also reported to occur in advanced stages of thyroid carcinoma, neuroblastoma and osteosarcoma. Although the expression of nm-23 is different in various types of human cancers, the decrease in expression is closely related to the increased metastatic potential in most types of carcinomas.

MTA-1 is a protein that is widely used for the prognosis and diagnosis of several types of human cancer and is closely related to the development of cancer, aggressive phenotype and poor prognosis in cancer patients. MTA-1 modulates the expression of target genes because of its ability to act as a corepressor or coactivator. MTA-1 targets and effector pathways regulate the mechanisms through cellular function, both in normal cells and cancer. It was reported that increased expression of MTA-1 indicates malignancy and aggressiveness in lung cancer, pancreas, colorectal, and prostate cancer. Although the two markers have shown their respective roles and functions in the process of invasion and metastasis, the molecular mechanism in Burkitt's lymphoma oral cancer cells is still unclear.

p27Kip-1 is a negative regulator of the cell cycle and a universal cyclin-dependent kinase inhibitor. p27 directly inhibits the activity of the cyclins-CDK enzyme causing cell cycle arrest in the G1 phase. In this study, p27 was transfected using oligonucleotides to target p27 sense. The aim of this study was to analyze the expression of nm-23 and MTA-1 proteins in Burkitt's lymphoma oral malignant cells induced by oligonucleotide p27 sense.

Materials and methods

Cells and cell culture
Raji cells were obtained from Department of Parasitology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. 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).

Sense experiments
Sense experiments were carried out as described previously. Two oligonucleotides for p27Kip-1 (Fasmac Co., Kanagawa, Japan) were synthesized as follows: S, 5'-GGCGCAGGAGCCA-3' and AS, 5'-TGGCTCTCTGCGCC-3' (the AS direction of human p27Kip-1 cDNA nucleotide, 15 mer). The oligonucleotides were delivered into Raji cell line directly according to the manufacturer's instructions.

Western blot analysis
Preparing cell lysates from the Raji-treated cells in tissue culture Ø 10 cm for 48 h. Briefly, samples containing equal amounts of protein (70 μg) were electrophoresis on a SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane (BioRad, Hercules, CA, USA). The filters were blocked in TBS containing 5% non-fat milk powder at 37°C for 1 h, and then incubated with a 1:500 dilution of primary antibodies against the p27Kip-1 protein (clone 1B4, mouse monoclonal antibody; Novocastra Laboratories, New Castle, UK), MTA-1 protein (AV37737, rabbit polyclonal; Sigma-Aldrich, USA), nm-23 (Santa Cruz Biotech, USA). For detection of HRP-conjugated antibodies were used the enhanced chemiluminescent (ECL) plus kit (Amersham Pharmacia Biotech, UK). Anti α-tubulin monoclonal antibody (Zymed laboratories, San Francisco, USA) was used for normalization of the western blot analysis.

Apoptosis induction assay using AO-EB staining
Microplate 6 wells was prepared and put in the cover glass at each well. Raji cells (2x10⁵ cells / wells) from the incubator were cultured in a well contained DMEM 10% fetal calf serum (FCS). Subsequently transfected cells were incubated for 24 hours at 37°C and C0 5%. After incubation, medium was aspirated and each well was given new DMEM 10% FBS. Cells were incubated for 48 hours. After that the glass cover at the bottom of the well was removed and placed on the object glass. All samples were stained with a combination of 10 μl ethidium
bromide-acridine orange solution by dropping on a glass cover for 10 minutes. All preparations were observed under a fluorescence microscope with magnification 40x. Apoptosis cells were measured by manual standard procedure (unit: %).

### Statistical analysis

Data was analyzed using one-way ANOVA followed by Post Hoc (LSD) with 95% significance level. The analysis program was carried out by SPSS 17.00 software (SAS Institute Inc, Cary, NC, USA).

### Results

#### Cell transfectants

Growth of Raji cells transfected with oligonucleotide sense and antisense p27 for 48 hours of incubation was shown in Figure 1.

![Figure 1. Growth of Raji cells transfected with oligonucleotide sense and antisense p27Kip-1 for 48 hours.](image)

Based on Figure 1, the growth of Raji-p27Kip-1 AS cells and control revealed rapid cell growth compared to Raji-p27Kip-1 S cells for 48 hours of incubation. In addition, the growth of Raji-p27Kip-1 AS cells was detected more rapid than control. In contrast, Raji-p27Kip-1 S cells indicated significant cell growth inhibition compared to p27Kip-1 AS and control. Interestingly, p27Kip-1 S was markedly effective in inhibiting the growth of Burkitt's lymphoma cancer cells.

#### Western blotting analysis

Western blot analysis was used to examine the protein expression of p27Kip-1, MTA-1, nm-23 and α-tubulin in Raji-transfected cells. As shown in Figure 2, up-regulation of p27Kip-1 and nm-23 protein was detected in Raji-p27Kip-1 S cells compared with that of p27Kip-1 AS and control. Furthermore, down-regulation of MTA-1 was obtained in p27Kip-1 S cells. However, increased expression of MTA-1 protein was found in p27Kip-1 AS cells. The expression of α-tubulin as an internal control was approximately the same in each Raji-transfected cells.

![Figure 2. Western blotting analysis. Expression of MTA-1, nm-23, p27Kip1 and α-Tubulin on Raji cell transfected with sense and antisense p27.](image)

#### Induction of apoptosis by double staining analysis

Apoptosis induction was carried out with a double staining using an AO-EB assay. Based on Figure 3, an increase in apoptosis occurs in Raji-p27Kip-1 S cells compared to control and Raji-p27Kip-1 AS cells. Cell apoptosis also occurs in Raji-p27Kip-1 AS cells, although only in a small part of cells. Furthermore, Raji cell transfected with p27Kip-1 S revealed a strong potency in suppressing the Burkitt's lymphoma cells through apoptosis mechanism.

### Discussion

The orderly transit of cells through the cell cycle requires a delicate balance between positive and negative regulatory factors. Any alterations in this balance can result in abnormal cell proliferation, which may contribute to cancer. Oral cancer is characterized by a high degree of local invasion and a high rate of metastasis to the cervical lymph nodes and distant 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. Despite advances in surgery, radiotherapy and chemotherapy, the survival of patients with oral cancer has not significantly improved over the past several decades. For this reason, gene therapy is
needed, one of which uses sense and antisense experiment include oligonucleotide p27 sense.

p27Kip-1 is a negative regulator of the cell cycle and a universal cyclin-dependent kinase inhibitor. p27Kip-1 is a putative tumor suppressor gene,\(^2\) a regulator of drug resistance in solid tumors,\(^2\) and a promoter of apoptosis.\(^2\) Moreover, p27Kip1 acts as a safeguard against inflammatory injury\(^2\) and plays a role in cell differentiation.\(^2\)

In the last decade, treatment strategy using gene transfection technique on human head and neck cancer included oral cancer has a main attention of clinicians or oncologists in the world. In the present study, transfection with p27 sense introduced into cultured Raji cells increased the cell growth suppression and induced apoptosis, in contrast to p27 antisense transfection. These results clearly showed that cell growth was inhibited by the p27 S effect and not by non-specific effect such as oligonucleotide toxicity. It has been reported that S and AS oligonucleotides hybridized to the complementary target mRNA and caused a steric or conformational obstacle for protein translation. As a result, the production of a specific protein is temporarily inhibited without affecting the expression of other genes and without intervention at the gene level.\(^2\) Dias and Stein\(^2\) reported that the mechanism of action of oligonucleotide can be discerned through the Rnase H-dependent oligonucleotide. These appear to induce the degradation of mRNA and the steric-blocker oligonucleotides, which physically prevent or inhibit the progression of splicing or the translational machinery. Interestingly, oligonucleotide-assisted Rnase H-dependent reduction of targeted RNA expression can be quite efficient, reaching 80-95% down-regulation of protein and mRNA expression.

As expected from the cell growth inhibitory effect (Figure 1), an increase in activation of proteolytic activity in p27 S-treated cells strongly suggest that apoptosis occurred in those cultures. Similar results were reported by Harada et al.\(^2\) who reported that in oral cancer, induction of apoptosis can be caused by up-regulation of p27Kip-1 and down-regulation of p45Skp-2 (or up-regulation of Kip-1 S and Skp-2 AS). In the present study, apoptotic induction was shown in many red color in Raji-p27 S cells compared to AS p27 and controls (Figure 3). Several investigators had already reported a relationship among p27Kip-1 and apoptosis in their experiments. Decreased expression of p27Kip-1 has been frequently detected in human cancer. Loss of p27Kip-1 has been associated with disease progression and an unfavorable outcome in several types of malignancy.\(^2\) Mice lacking the p27Kip-1 gene show an increase in body weight, thymic hypertrophy and hyperplasia of pituitary intermediate lobe adrenocorticotropic hormone cells, adrenal glands and gonadal organ.\(^2\) Also, malignant human oral cancer cells transfection with p27Kip-1 gene leads to inhibition of proliferation, invasion and metastasis.\(^1\)

Furthermore, expression of MTA-1 and induced nm-23 and p27Kip1 proteins were detected in Raji-p27Kip-1 S. These suggested transfection of oligonucleotide sense to p27Kip-1 was markedly suppressed the MTA-1 protein, a main biomarker of metastasis and target therapy of p27Kip-1 S gene. It was reported overexpression of MTA-1 protein indicated high proliferation, invasion, metastasis and recurrence in gastric cancer,\(^2\) and also showed rapid formation of tumor angiogenesis and poor survival in lung cancer.\(^2\) However, in the present study we detected down-regulation of MTA-1 protein level through Western blotting analysis in cell transfected with p27Kip-1 S.

**Conclusions**

In conclusion, oligonucleotide p27Kip-1 S has a strong antitumor activity in oral malignant Burkitt’s lymphoma through increasing the apoptosis activity, expression of nm-23 and p27Kip-1 induction, and also suppressing the expression of MTA-1 protein targeting this molecule could represent a promising new therapeutic approach for this type of cancer.

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Declaration of Interest

The authors report no conflict of interest.

References