

# Up-Regulation of S-Phase Kinase Associated Protein-2 Antisense Induces Cell Growth and Migration Chemotactic Suppression and Apoptosis in a Malignant Oral Burkitt's Lymphoma Cells

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**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. One of method for high-efficiency and region-controlled *in vitro* gene transfer was developed by antisense oligonucleotide. In the present study, Skp2 sense (p45<sup>Skp2</sup> S), Skp2 antisense (p45<sup>Skp2</sup> AS) and Skp2 neo (empty vector) were transfected into an oral Burkitt's lymphoma (Raji) cell line. The aim of study was to examine the Skp2 AS up-regulation toward the cell growth and migration chemotactic suppression and apoptosis induction in an oral Burkitt's lymphoma cells *in vitro*. The efficiency of Skp2 S and Skp2 AS gene transfection in cell growth inhibition test was confirmed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. To estimate the suppression of chemotactic migration cell was examined by Boyden chamber assay and cell growth test. Furthermore, colorimetric assay caspase-3 and caspase-9 were used to know the apoptosis induction. Results revealed the Raji cell growth and migration chemotactic were markedly suppressed by Skp2 AS. Skp2 AS-treated cell induced apoptosis characterized by an increase caspase-3 and -9 proteolytic activities. These results suggest that up-regulated of Skp 2 AS appear to induce cell growth and migration chemotactic suppression and apoptosis induction in Raji cells targeting this molecule could represent a promising new therapeutic approach for this type of tumor.

**Keyword:** cell growth, Skp2 AS, Burkitt's lymphoma cell, apoptosis, migration.

## 1. Introduction

S-phase kinase associated kinase 2 (Skp2), a member of the F-box family, is the substrate-recognition subunit of the SCF<sup>Skp2</sup> ubiquitin ligase complex.<sup>1)</sup> Skp2 has been implicated in ubiquitin-mediated degradation of the cyclin-dependent kinase (CDK) inhibitor p27<sup>Kip1</sup>, and positively regulates the G<sub>1</sub>/S transition.<sup>2-4)</sup> The targeted disruption of Skp2 leads to the accumulation of p27 and cell cycle arrest in G<sub>1</sub>. Moreover, Skp2 has also been required for ubiquitination of other cell-cycle regulators including free cyclin E<sup>5)</sup>, E2F1<sup>6)</sup> and hOrclp.<sup>7)</sup> Overexpression of Skp2 has been observed in various type of human tumors. Elevated expression of Skp2 indicates poor prognoses for patients with colorectal,<sup>8)</sup> lymphoma,<sup>9)</sup> gastric,<sup>10)</sup> and lung cancers.<sup>4)</sup> Therefore, Skp2 knock-out mice grow more slowly and have smaller organs than littermate controls, and they show a reduced growth rate and increased apoptosis.<sup>5)</sup> However, the mechanism of Skp2 overexpression in cancer cells or the nature of its contribution to the malignant phenotype is little known.

Burkitt's lymphoma (BL) is one of the most aggressive malignancies of lymphoid origin and accounts for 3-5% of all lymphomas. It is a high grade B-cell neoplasm. Usually found in the pediatric population, BL represents 40% of childhood NHL<sup>11)</sup>. The highest incidence is found in the endemic form in equatorial regions of Africa and Papua-New Guinea where it accounts for 50-70% of all pediatric malignancies<sup>12)</sup>. BL is characterized by chromosome

translocations between the proto-oncogene c-myc and one of the immunoglobulin (Ig) loci<sup>13)</sup>. Epstein-Barr virus (EBV) has been implicated in its etiology. BL most often involves the maxilla or the mandible and rarely involves abdominal organs such as kidneys, ovaries or retroperitoneal structures. In the present study, up-regulation of Skp2 AS toward the cell growth and migration chemotactic suppression and apoptosis induction in an oral Burkitt's lymphoma cells *in vitro* were examined.

## 2. Materials and Methods

### Cell and cell culture

Raji cell line (ATCC CCL-86) was obtained from Department of Paracytology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. Cell line was cultured in Dulbecco's modified Eagle medium (Sigma-aldrich, St Louis, MO, USA) supplemented with 10% fetal calf serum, and 100 µg/ml streptomycin, 100 U/ml penicillin (Moregate BioTech, Bulimba, Australia). The cultures were incubated in humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C.

### Antisense experiments

AS experiments were performed as described previously.<sup>14)</sup> Two oligonucleotides containing phosphorothioate backbones (Fasmac, Kanagawa, Japan) were synthesized as follows: AS, 5'-TCCTGTGCATAGCGTCCGCAGGCC-3' (AS direction of human Skp2 cDNA nucleotides 15 to 25), SC, 5'-CCCGGACGCCTGCGATACGTGTCTCT-3'

(SC for AS).<sup>4)</sup> The oligonucleotides were delivered into Raji cell line directly according to the manufacturer's instructions.

#### MTT assay

Raji cell transfected with AS, S and untreated control (UC) were seeded on 96-well plates (Falcon, NJ, USA) at  $1 \times 10^4$  cells per well in DMEM containing 10% FCS, the day before treatment. Cell line was treated with oligonucleotides at final concentration 100  $\mu$ M. After 24 hours, the number of cells was quantitated by an assay in which MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-aldrich) was used.<sup>15)</sup>

#### 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 Raji cell line transfected with Skp2 AS, S 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.<sup>14)</sup>

#### Chemotactic migration assay

Chemotaxis (directed migration) was evaluated in the Boyden chamber apparatus (Neuro Probe, Inc., Cabin John, MD, USA). Briefly, subconfluent cells were starved for 24 h and harvested with 0.05% (w/v) trypsin (Invitrogen Corporation, USA) containing 0.02% (w/v) ethylenediamine tetra-acetic acid (EDTA, Invitrogen Corporation), washed twice with PBS, and resuspended to a final concentration of  $5 \times 10^5$  per ml in serum-free medium with 0.1% (w/v) fraction V bovine serum albumin (BSA, Wako Pure Chemical Industries, Ltd). Polyvinyl-pyrrolidone (PVP) filters (Nuclepore Corp, Palo Alto, CA, USA) of 8- $\mu$ m pore size were precoated with gelatin (Merck KGaA, Frankfurt, Darmstadt, Germany) (0.1 mg/ml) and rinsed in sterile water. Lower chamber were filled with 30  $\mu$ l of 10% FBS in DMEM and covered with a gelatin-coated membrane. Furthermore, 50  $\mu$ l of cell suspension, yielding 500 cells/ml of Raji-Skp2 AS, Raji-Skp2 S, Raji-UC were added to the upper chamber. After 24 hours of incubation, the membrane was stained with Giemsa solution (Ted Pella Inc., Redding, CA, USA). The number of cells that had penetrated through the filter was counted under light microscope at 400x magnification. The counting was performed for 12 fields in each concentration.

#### Statistical analysis

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

### 3. Results

#### Growth of cells *in vitro*

In this examination, the effect of oligonucleotides AS, S or UC at final concentration 100  $\mu$ M on *in vitro* cell growth of Raji cells was evaluated.<sup>16)</sup> Relative cell number was evaluated by comparing the absorbance in each cell at 24 hours. The cell number of AS-treated cells was significantly decreased the growth of Raji cells when compared with that of S- and UC-treated cells (P=0.015) (Figure 1).

#### Chemotactic migration assay

Cell migration is an essential process involved in tumor invasion and metastasis.<sup>16)</sup> The chemotactic ability of each transfection cells with Boyden chamber kit was evaluated for 24 h incubation. As seen in Figure 2A, Raji-Skp2 AS showed significantly lower ability of chemotactic migration compared with that of the non-treated Raji cells (P=0.001). Figure 2B indicated Raji-Skp2 AS was markedly inhibited the cell growth when compared with Raji-Skp2 S.

#### Proteolytic activities of caspase-3 and -9

The activity of caspase-3 and -9 in Raji-Skp2 AS, Raji-Skp2 S and Raji-UC were investigated. Raji-Skp2 AS showed increased caspase-3 and -9 proteolytic activities as compared with that of Skp2 S or UC (Figure 3). Proteolytic activities of caspase-3 in Raji-Skp2 AS was 2.9 fold increased compared with UC. Furthermore, proteolytic activities of caspase-9 in Raji-Skp2 AS was 1.6 fold increased (P = 0.001).

### 4. Discussion

The normal operation of cell cycle requires a delicate balance between positive and negative regulatory factors. Any alteration in this balance can result in abnormal cell proliferation, which may lead to malignant transformation. Loss of normal cell cycle control plays a crucial role in the genesis of most types of cancer.<sup>18)</sup> S-phase kinase-associated protein 2 was described as an F-box protein constituting the substrate-recognition subunit of the SCF<sup>Skp2</sup> ubiquitin ligase complex which targets cell-cycle regulator such as the CDK inhibitor p27Kip1, for ubiquitin-mediated degradation.<sup>4)</sup> A number of studies have found high levels of Skp2 expression, and its inverse correlation with p27Kip1 levels in various types of human tumors have associated with high aggressiveness and poor prognosis.<sup>19-20)</sup> In the present study, the phosphorotioate oligonucleotide AS strategy was employed to investigate the activity of Skp2 on cell growth and migration chemotactic suppression and apoptosis induction in a malignant oral Burkitt's lymphoma cell line.

Transfection with AS introduced into cultured Raji cells increased the cell growth and migration chemotactic suppression effect, in contrast to S or UC transfection. These results clearly showed that cell growth and migration chemotactic was inhibited by the AS effect and not by non-specific effect such as oligonucleotide toxicity. It has been reported that 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.<sup>21)</sup> Dias and Stein<sup>22)</sup> reported that the mechanism of action of AS 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 and migration chemotactic inhibitory effect, an increase in activation of caspase-3 and -9 in AS-treated cells strongly suggest that apoptosis occurred in those cultures. Also, in the current study, increased caspase-3 and -9 activation in AS-treated cells revealed that apoptosis ensued extrinsic and intrinsic pathways. Similar results were reported by Harada *et al.*<sup>22)</sup> who suggested that in oral cancer, induction of apoptosis can be caused by down-regulation of Skp2 (or up-regulation of Skp2 AS) .

Several investigators had already reported a relationship between Skp2 and apoptosis in their experiments. S-phase induction by adenovirus-vector mediated expression of Skp2 in quiescent cells was followed by apoptosis.<sup>4)</sup> Mice embryonic fibroblasts in Skp2-deficient mice showed an increased tendency toward spontaneous apoptosis.<sup>5)</sup> However, the actual role of Skp2 in apoptosis remains unclear. In the present study, Skp2 AS led to apoptosis characterized with induction of caspase-3 and -9 activities.

In conclusion, up-regulation of Skp2 AS could induce cell growth and migration chemotactic suppression and apoptosis in a malignant oral Burkitt's lymphoma (Raji) cell line. In addition, since component of apoptotic programs represent promising targets for anticancer therapy, up-regulation of Skp2 AS approach could be a useful apoptosis-modulating strategy for the treatment of several human cancers.

## 5. Acknowledgments

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## 6. Conflict of interest

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

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**Figure Legend**

Figure 1. Relative cell number was evaluated by comparing the absorbance in each cell at 24 hours using MTT assay. Values shown are the mean of six determinations; error bars indicate standard deviations.

Figure 2. A. Analysis of chemotactic migration ability of Skp2 AS, S and UC in Raji cells was examined by Boyden chamber assay. Raji cells treated with Skp2 AS for 24 hours showed significantly lower ability of chemotactic compared with that of the S or UC treated cells. Values are shown as mean; error bars indicate standard deviation. B. The growth of Raji cell transfected with Skp 2 S and AS for 48 hours.

Figure 3. The activity of caspase-3 and -9 in Raji cell line. Cells were treated with Skp2 AS, Skp2 S or UC oligonucleotides. \*\* Values are shown as mean; error bars indicate standard deviation.

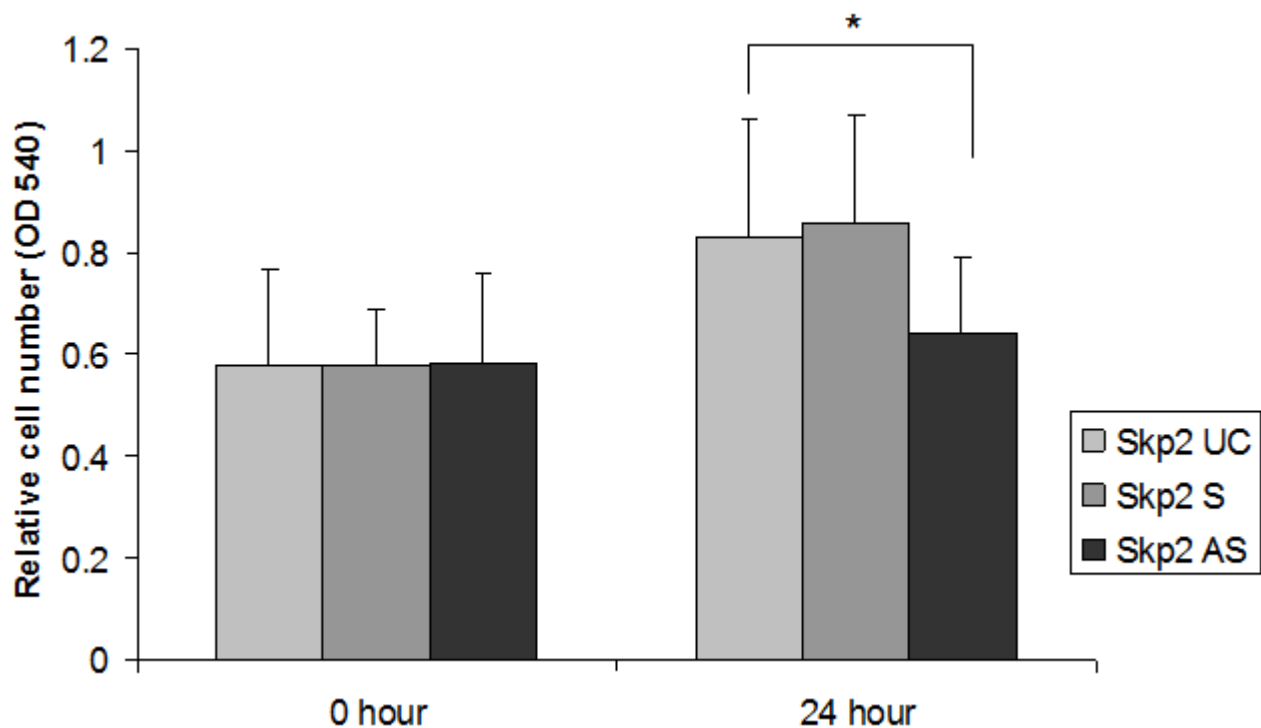


Figure 1

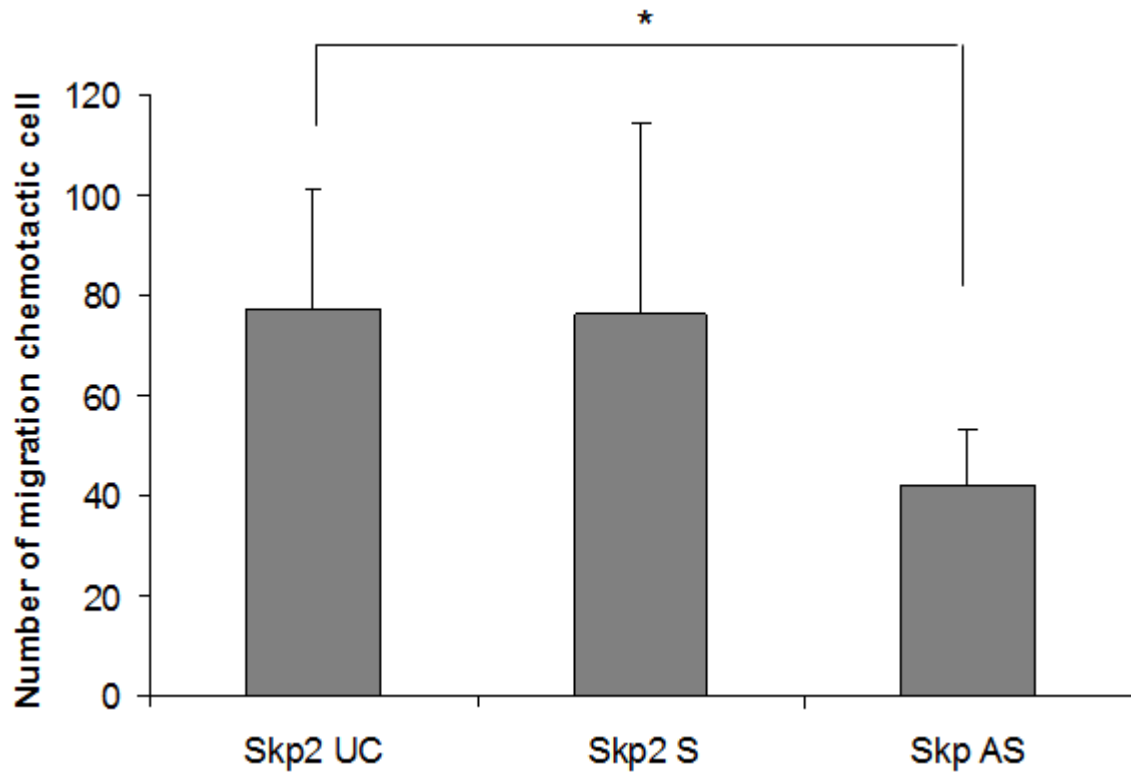
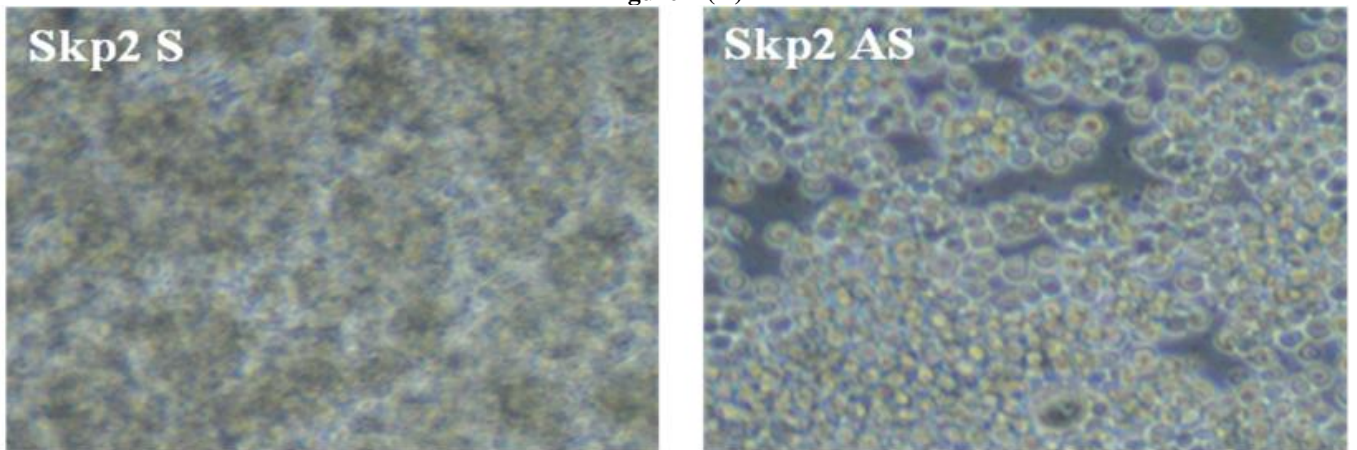
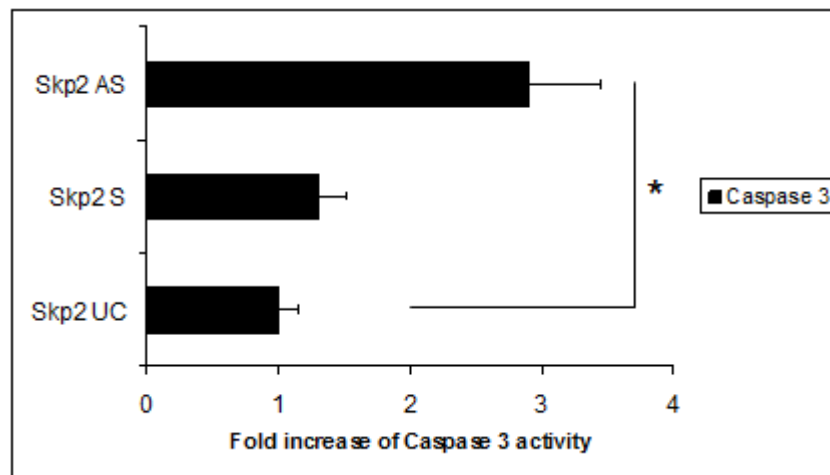


Figure 2 (A)



B.



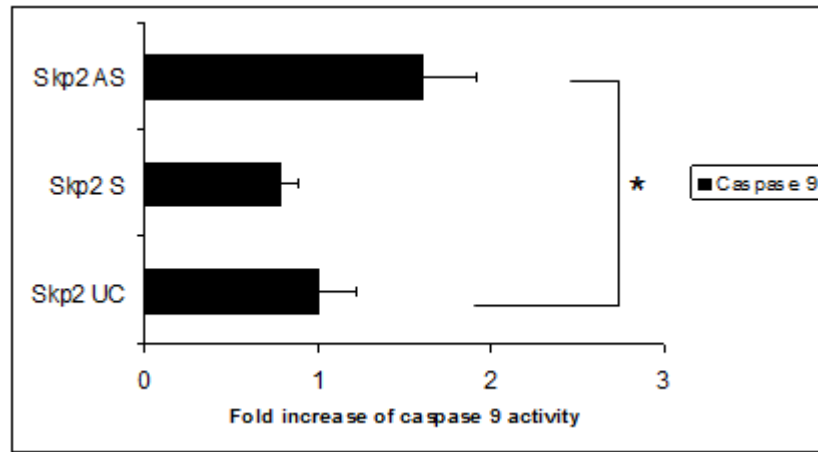


Figure 3