Antisense oligonucleotide p45Skp-2 suppresses migratory chemotactic and metastasis of oral malignant Burkitt's lymphoma cell through down-regulation of MTA-1 and induction of E-cadherin mechanism

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ABSTRACT

Introduction: Burkitt's lymphoma is a high-grade B-cell neoplasm and one of the most aggressive malignancies of lymphoid origins which found mainly in the paediatric population. The treatment options of this tumour are still limited. However, a new strategy for refractory tumour, phosphorothioate oligonucleotide antisense technique has watched with keen interest. This study was aimed to examine the effect of antisense p45Skp-2 (Skp-2 AS) suppressed migratory chemotactic and metastasis of oral malignant Burkitt's lymphoma (Raji) cell through down-regulation of MTA-1 and E-cadherin. **Methods:** True experiment laboratory with post-test control group design was confirmed in this study. The efficiency of Skp-2 AS in the suppression of cell chemotactic migration was examined by Boyden chamber assay. To evaluate the inhibition of cell metastasis was conducted by decreasing MTA-1 expression protein. The expressions of MTA-1, E-cadherin and α-tubulin protein were investigated by Western blot analysis. **Results:** The results revealed that the number of chemotactic migration of Skp-2 AS treated Raji cell was significantly decreased when compared with that of sense p45Skp-2 (Skp-2 S) and scrambled control (SC) cells ($P<0.05$) followed by decreased expressions of MTA-1 protein and overexpression of E-cadherin. Interestingly, the expression of α-tubulin protein as an internal control was approximately similar in each transfectant cells. **Conclusion:** p45Skp-2 have an antitumor activity via suppression of migratory chemotactic activity and metastasis on oral Burkitt's lymphoma cells through down-regulation of MTA-1 and induction of E-cadherin proteins targeting this molecule could represent a promising new therapeutic approach for this type of cancer.

Keywords: Burkitt's lymphoma cell, Skp-2 AS, MTA-1, E-cadherin, chemotactic migration, metastatic.

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INTRODUCTION

S-phase kinase-associated protein-2 (p45Skp-2 or Skp-2) is a ubiquitin ligase complex of Skp, Cullin, F-box (SCF)-Skp-2.[1] Skp-2 plays a role in the ubiquitin-mediated degradation of the cyclin-dependent kinase (CDK) inhibitor p27Kip1 and positively regulates the G_1-to-S transition.[2,3] Decreased Skp2 expression leads to accumulation of p27 and induces cell cycle arrest in the G_1 phase. Skp2 is required by other cell-cycle regulators, such as free cyclin-E,[4] E2F1,[5] and hOrclp, for ubiquitination [6]. Up-regulation of Skp-2 has been observed in various types of human cancer. Overexpression of Skp-2 indicates a poor prognosis and aggressive disease among patients with colorectal,[7] hypupharingeal[8], gastric,[9] and pancreatic cancers[2] Skp2 knock-out (-/-) mice show smaller organs and slower growth, and their cells exhibit induced apoptosis.[4] Despite these findings, however, little is known about the mechanism of Skp-2 overexpression in cancer cells or the nature of the contribution of this protein to the malignant phenotype.

Burkitt's lymphoma (BL) is a high-grade and aggressive malignancy of B-cell lymphoid origin that occurs as 3%–5% of all lymphomas. BL occurs in many groups of children, 40% of whom suffer from non-Hodgkin's lymphoma.[10] The equatorial areas of Africa and Papua New Guinea have the highest incidence of all pediatric malignancies (50%–70%).[11] BL is characterized by chromosomal translocations between c-myc and one of the immunoglobulin loci.[12] The Epstein-Barr virus has been reported to induce the aggressiveness of BL cells. In the oral cavity, BL occurs most often at the maxilla or the mandible, although other areas may also be affected. Thus, development of an effective therapeutic approach for oral malignant BL is an urgent undertaking. Unfortunately, studies on oral malignant BL are limited, and the disease remains poorly understood.

It was reported that the roles of Skp-2 as a positive regulator of cell cycle in the human tumours, included Burkitt's lymphoma, are to promote the cell growth, induce the cell chemotactic migratory and suppress the cell apoptotic.[14] Skp-2 protein as a co-activator of kinase inhibitor protein-1 (KIP-1) increases the cell invasion and metastatic through down-regulation of p27Kip-1.[16] The low accumulation of p27Kip-1 and high expression of Skp-2 protein reveal the cell aggressiveness, poor prognosis, and high mortality.[14] The effect of Skp-2 AS on an oral malignant Burkitt's lymphoma (Raji) cell through suppression of cell migratory chemotactic and metastatic with down-regulation of metastasis associated protein-1 (MTA-1) and E-cadherin mechanism was investigated. Genetic engineering of Antisense was carried-out to reverse the main function of Skp-2 protein. The aim of study was to examine the effect of antisense p45Skp-2 (Skp-2 AS) suppressed migratory chemotactic and metastasis of oral malignant Burkitt's lymphoma (Raji) cell through down-regulation of MTA-1 and E-cadherin.

METHODS

The true experimental laboratory with post-test only control group design was confirmed as a type and design of research. This study was approved by the research ethics committee of the Faculty of Dentistry Gadjah Mada University with no. 001385 / KKEP / FKG-UGM / EC / 2018.

Cells and cell cultures

Raji cells (ATCC CCL-86 B-lymphocyte, USA) were obtained from the Department of Parasitology, Faculty of Medicine Gadjah Mada University. The cells were cultured in Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich, USA) supplemented with 10% fetal calf serum, 100 g/mL streptomycin, and 100 µg/mL penicillin (Moregate BioTech, Australia). The cultures were incubated at 37°C with 5% CO_2 in a 95% humidified atmosphere.

Antisense oligonucleated experiments (AS)

AS experiments were performed as described previously.[13] Two oligonucleotides containing phosphorothioate backbones were synthesized as follows: AS, 5'-TCCTGTGCAT AGCGTCCGCAGGCCC–3' (the AS direction of human Skp2 cDNA nucleotide 22-46)[13], S, 5'-CCCGGACGCCTGCGATACGTGTCCT-3' (S for AS)[13]. The oligonucleotides were delivered into Raji cell line directly according to the manufacturer's instructions (Antisense technology, Ionis Pharmaceutical, Inc, USA).

Migration chemotactic assay by Boyden chamber kit

The Raji cell line (5 x 10^5 cell/kit) was seeded and placed in the upper compartment (each well was 50 l) and was allowed to migrate through the pores of the membrane into the lower compartment, in which the chemotactic agents [medium + p45Skp-2 AS or p45Skp-2 S or control (SC)] were present. After an appropriate incubation time (approximately 24 hours), the membrane between the two compartments was fixed with pure methanol and stained with hematoxylin solution, and the number of cells that have migrated to the lower side of the membrane was determined using a light microscope with 40x magnitude.

Western blotting analysis

The cell lysates were prepared from the Raji-treated cells in a Falcon tissue culture for 48 hours. Briefly, samples containing equal amounts of protein (70 µg; using standard procedure for measuring hole protein) were performed an electrophoresis on a SDS-polyacrylamide gel and transferred to a polyvynilidene fluoride (PVDF) membrane (BioRad, Hercules, CA, USA). The filters were blocked in the Trys buffered saline (TBS) containing 5% non-fat milk powder at 37^{0}C for 1 hour, and then incubated with a 1:500 dilution of primary antibodies against the p45Skp-2 protein (H-435, rabbit polyclonal antibody; Santa Cruz Biotech, USA), MTA-1 protein (AV37737, rabbit polyclonal; Sigma-Aldrich, USA), CDK-2 (E304, rabbit monoclonal antibody, Abcam, USA) and cyclin E protein (clone HE12, mouse monoclonal antibody, Santa Cruz Biotech, USA).

Detection of HRP-conjugated antibodies was conducted using the enhanced chemiluminescent (ECL) plus kit (Amersham Pharmacia Biotech, UK). Anti-tubulin monoclonal antibody (Zymed laboratories, San Francisco, USA) was used for normalisation of the western blot analysis.

Statistical analysis

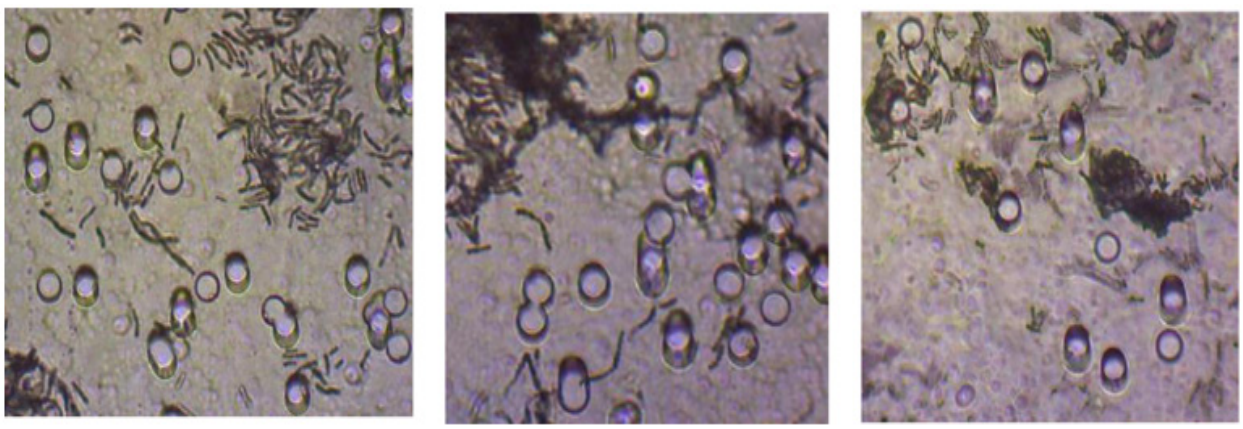
Statistical differences between the means for different groups were evaluated with Stat View 4.5 (Abacus Concepts, Berkeley, CA) software, using the one-way ANOVA test. The significance level was set at 5% for each analysis.

RESULTS

Chemotactic migration activity

The ability of cell migration on each transfected cell was evaluated for 24 h incubation. Raji-treated cells with p45Skp-2 AS (marked) showed the low ability of cell migration compared with the Skp-2 S and control ($p<0.05$) (Figure 1A and 1B).

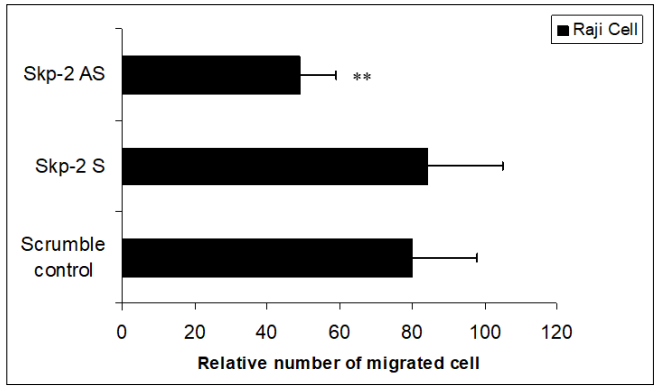
A.



Control (SC) **Raji-p45Skp-2 S** **Raji-p45Skp-2 AS**

Figure 1. Chemotactic migration activity of Raji cells transfected by oligonucleotides sense (S) and antisense (AS) Skp-2 for 24 hours

B.



A. Decreased number of Raji cells transfected with Skp-2 AS
B. Relative migrated cell number of Skp-2 AS, S and SC (; p=0.001, one-way ANOVA**

Western blotting analysis

Western blot analysis was used to examine the protein expression of Skp-2, MTA-1, E cadherin and α-tubulin in Raji-transfected with Skp-2 AS, S or SC. As shown in Figure 2, up-regulation of Skp-2 and E-cadherin protein was detected in Raji-Skp-2 AS cells compared with that of Skp-2 S. Furthermore, down-regulation of MTA-1 protein was obtained in Skp-2 AS cells. Interestingly, the expression of α-tubulin as an internal control was approximately the same in each Raji-transfected cells.

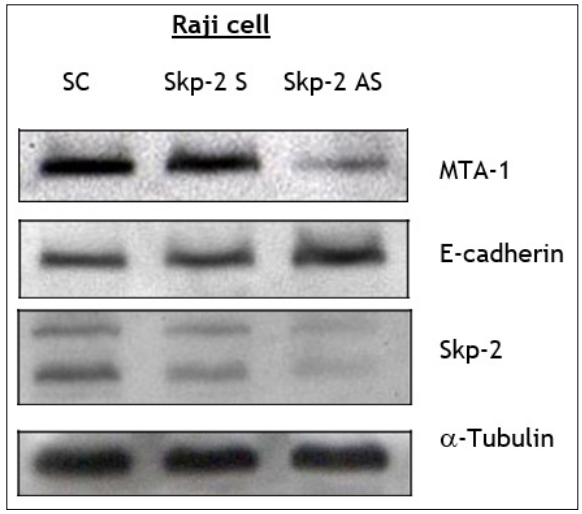


Figure 2. Protein expression of Skp-2, MTA-1, E-cadherin and α-tubulin in Raji cells transfected with Skp-2 AS, S or SC by western blotting analysis

DISCUSSION

Oligonucleotide-based therapies are advanced novel interventions used in the management of various diseases. Oligonucleotides can be used to modulate gene expression via a range of processes including RNAi, target degradation by RNase H-mediated cleavage, splicing modulation, non-coding RNA inhibition, gene activation and programmed gene editing. The use of oligonucleotides allows for precision and/or personalized medicine approaches. It can theoretically be designed to selectively target any gene with minimal, or at least predictable, off-target effects. Antisense oligonucleotides (AS-ODN) have small (18–30) nucleotides, synthetic, single-stranded nucleic acid polymers of diverse chemistries, which can be employed to modulate gene expression via various mechanisms.[13]

In this study, we used phosphorothioate oligonucleotide antisense to Skp-2 because of the function and role of Skp-2 as the tumor promoter gene or protein. Skp2 as a positive regulation of cell cycle can promote the cell migration or invasion. A number of studies have found high levels of Skp2 expression, and its inverse correlation with

$p27^{Kip1}$ level have been observed in lymphomas [8], oral squamous cell carcinomas [3,16] , lung cancer[2] and gastric carcinoma.[9] In the present study, the phosphorotioate oligonucleotide AS and S strategy was delivered to investigate the activity of Skp-2 on migratory chemotactic and metastasis suppresion in an oral malignant Burkitt's lymphoma cell line (Raji cells) through the down-regulation of MTA-1 and induction of E-cadherin expression. Transfection with Skp-2 AS into Raji cells increased the migratory chemotactic suppression effect (Figure 1 and 2).

These results clearly revealed that cell migratory chemotactic was inhibited by Skp-2 AS effect and not by non-specific effect such as oligonucleotide toxicity.[2] 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 spesific protein is temporarily inhibited without affecting the expression of other genes and without intervention at the gene level.[14]

Recently, it was 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.[15] 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.[15] Recent study reported the relationship between down-regulation of Skp-2 with apoptosis. S-phase kinase protein-2 induction by adenovirus-vector mediated expression of Skp-2 in quiescent cells was followed by apoptosis.[2] Mice embryonic fibroblasts in Skp-2 deficient mice showed an increased tendency toward spontaneous apoptosis.[4]

However, the actual role of Skp-2 in apoptosis remains unclear. It has been reported that the molecular mechanisms of Skp-2 have an inverse correlation with Kip-1.[15] Up-regulated of Skp-2 or functional loss of Kip-1 has been implicated in poor prognosis, high carcinogenesis and cancer progression.[3] In the current research, the mechanism of antisense Skp-2 in suppressing the chemotactic migration, cell growth, metastatic and down regulation of apoptosis via biomolecular mechanism, which can explain why the Skp-2 can promote cel growth and Skp-2 antisense can suppress cell growth.

As expected from the migratory chemotactic inhibitory effect, an increase in protein expression of Skp-2 and E-cadherin was detected in Raji-Skp-2 AS cells strongly suggest that suppression of this migratory cell occured through the target molecule Skp-2 and E-cadherin. It was reported that the loss of E-cadherin expression in association with the epithelial–mesenchymal transition (EMT) occurs frequently during tumor progression, invasion and metastasis.[21] E-cadherin is particularly active area of research in development and tumorigenesis.

The calcium-dependent interactions among E-cadherin molecules are critical for the formation and maintenance of adherent junctions in areas of epithelial cell-cell contact. Loss of E-cadherin-mediated-adhesion characterises the transition from benign lesions to invasive and metastatic cancer. The mechanism that renders E-cadherin functional is unknown, but it does include phosphorylation of the protein.

Controlled epithelial-mesenchymal conversion is the most important exhibit of E-cadherin's function in development.[22] Furthermore, the expression of MTA-1 protein was markedly decreased in Raji-Skp-2 AS. This data strongly suggest that Skp-2 AS can suppress Raji cell invasion and metastasis. It was reported The MTA-1 protein contributes to the process of cancer progression and metastasis.[17] overexpression of MTA-1 protein indicated high proliferation, invasion, metastasis and recurrence in gastric cancer,[18-19] and also showed rapid formation of tumor angiogenesis and poor survival in lung cancer.[20]

Based on the findings of our 3-years research (2017-2019), we recommend continuing this research by examining other anticancer activities including cell apoptosis, cell cycle arrest, tumirigenesis assay, miRNA assay and in vivo tests. We also have research limitations in the form of research materials that must be obtained abroad and take a long time to be available, research instruments that were limited and queued up, and complicated research permit correspondence.

CONCLUSION

p45Skp-2 have an antitumor activity via suppression of migratory chemotactic activity and metastasis on oral Burkitt's lymphoma cells through down-regulation of MTA-1 and induction of E-cadherin proteins targeting this molecule could represent a promising new therapeutic approach for this type of cancer.

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