

## Research Report

## Antitumor activity of antisense oligonucleotide p45<sup>Skp2</sup> in soft palate carcinoma cell squamous in vitro

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### ABSTRACT

**Background:** Human soft palate cancers are characterized by a high degree of local invasion and metastasis to the regional lymph nodes. Treatment options for this cancer are limited. However, a new strategy for refractory cancer, gene therapy is watched with keen interest. p45<sup>Skp2</sup> gene as a tumor promoter gene is one of target of the oral cancer therapy. To inhibit the activity of p45<sup>Skp2</sup> gene is carried-out the genetic engineering via antisense technique. **Purpose:** To examine the antitumor activity of p45<sup>Skp2</sup> antisense (p45<sup>Skp2</sup> AS) gene therapy in human soft palate [Hamakawa-Inoue (HI)] cancer cells. **Methods:** Pure laboratory experimental study with post test only control group design was conducted as a research design. To investigate the apoptosis induction of p45<sup>Skp2</sup> AS-transfected cell was evaluated by colorimetric caspase-3 assay and Flow cytometry. Furthermore, to detect the suppression of in vitro HI cell invasion and cell growth of p45<sup>Skp2</sup> AS-treatment cell was examined by Boyden chamber kit and MTT assay, respectively. **Results:** The cell number of p45<sup>Skp2</sup> AS-treated HI cell was significant decreased when compared with that of p45<sup>Skp2</sup> sense (p45<sup>Skp2</sup> S) cells ( $p < 0.05$ ). p45<sup>Skp2</sup> AS-treated cell induced apoptosis characterized by an increase in the early and late apoptosis, and activation of caspase-3 ( $p < 0.05$ ). Therefore, suppression of HI cell invasion and cell growth were markedly increased by p45<sup>Skp2</sup> AS treatment ( $p < 0.05$ ). **Conclusion:** Antisense oligonucleotide p45<sup>Skp2</sup> has a high antitumor activity in human soft palate cancer cell, targeting this molecule could represent a promising new therapeutics approach for this type of cancer.

**Key words:** Antisense p45<sup>Skp2</sup> gene, apoptosis, invasion, soft palate cancer cell

### ABSTRAK

**Latar belakang:** Kanker palatum lunak mempunyai karakteristik invasi dan metastasis ke limfonodi regional yang tinggi. Pilihan perawatan kanker tersebut masih sangat terbatas. Walaupun demikian, strategi baru untuk penanganan kanker yaitu terapi gen menjadi pilihan utama. Gen p45<sup>Skp2</sup> sebagai gen pemacu tumor merupakan salah satu target terapi kanker oral. Untuk menghambat aktivitas gen p45<sup>Skp2</sup> tersebut dilakukan rekayasa genetik melalui teknik antisense. **Tujuan:** Menguji aktivitas antitumor gen p45<sup>Skp2</sup> antisense (p45<sup>Skp2</sup> AS) terhadap sel kanker palatum lunak (sel HI). **Metode:** Jenis penelitian yang digunakan adalah eksperimen laboratorium murni dengan rancangan posttest only control group design. Induksi apoptosis sel yang ditransfeksi p45<sup>Skp2</sup> AS dievaluasi menggunakan uji caspase-3 kolorimetrik dan flow cytometry. Untuk mendeteksi hambatan invasi dan pertumbuhan sel HI yang ditransfeksi p45<sup>Skp2</sup> AS dilakukan uji Boyden chamber dan uji MTT. **Hasil:** Pertumbuhan sel HI yang ditransfeksi p45<sup>Skp2</sup> AS menurun secara signifikan dibandingkan dengan p45<sup>Skp2</sup> Sense (S) ( $p < 0,05$ ). Sel HI transfeksi p45<sup>Skp2</sup> AS menginduksi apoptosis dengan meningkatkan aktivitas proteolitik caspase-3 dan early and late apoptosis ( $p < 0,05$ ). Hambatan invasi dan pertumbuhan sel HI secara signifikan meningkat pada sel yang diperlakukan dengan p45<sup>Skp2</sup> AS ( $p < 0,05$ ). **Kesimpulan:** p45<sup>Skp2</sup> AS oligonukleotida mempunyai aktivitas antitumor yang kuat pada sel kanker palatum lunak. Target dari molekul tersebut dapat menjanjikan suatu terapeutik baru untuk jenis kanker palatum tersebut.

**Kata kunci:** Gen p45<sup>Skp2</sup> AS, apoptosis, invasi, sel kanker palatum lunak

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## INTRODUCTION

Soft palate cancer is a rare cancerous growth of the mouth. Cancer of the soft palate accounts for approximately 2% of head and neck mucosal malignancies and most cancer of the palates are squamous cell carcinoma (SCC).<sup>1</sup> Human soft palate cancers are characterized by a high degree of local invasion and a high rate of metastases to the cervical lymph nodes. Moreover, human soft palate cancer frequently shows local recurrence after initial treatment, probably due to micro invasion and/or metastasis of tumor cells at the primary site.<sup>2</sup>

Despite advanced in surgery, radiotherapy and chemotherapy, the survival of patients with oral cancers include soft palate cancer has not significantly improved over the past several decades. Also, treatment options for recurrent or refractory soft palate cancers are limited.<sup>3</sup> Furthermore, the ratio of mortality in 1980 and 1990 was 48% and 47% respectively,<sup>4</sup> and the oral cancer prognosis has not changed during the past 10 years. However, as a new strategy for refractory cancer, gene therapy is watched with keen interest.

p45 S-phase kinase associated protein 2 (p45<sup>Skp2</sup> or Skp2), a member of the F-box family, is the substrate-recognition subunit of the SCF<sup>Skp2</sup> ubiquitin ligase complex.<sup>5</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>6</sup> The targeted disruption of Skp2 leads to the accumulation of p27 and cell cycle arrest in G<sub>1</sub>. Over expression of Skp2 has been observed in various types of human tumors. Elevated expression of Skp2 indicates poor prognoses for patients with colorectal,<sup>7</sup> lymphoma,<sup>8</sup> gastric,<sup>9</sup> and lung cancers.<sup>10</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>11</sup> However, the role of Skp2 over expression in cancer cells or the nature of its contribution to the malignant phenotype is little known.

In the present study, p45<sup>Skp2</sup> was combined with antisense oligonucleotide. This antisense is designed to hybridize to their specific mRNA target. The hybrid formation causes a steric block or conformational obstacle for protein translation. Antisense oligonucleotides are used to knock down protein expression by inhibiting the translation of the mRNA of a desired target gene.<sup>12</sup> The main purpose of the study was to examine the antitumor activity of p45<sup>Skp2</sup> antisense (p45<sup>Skp2</sup> AS) gene therapy in human soft palate [Hamakawa-Inoue (HI)] cancer cells.

## MATERIALS AND METHODS

Derivation of the human soft palate cancer cell line (HI) has been described.<sup>13</sup> HI cell line was cultured in Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich, USA) supplemented with 10% fetal calf serum (FCS, Moregate BioTech, Bulimba, Australia), and 100 µg/ml streptomycin, 100 U/ml penicillin (Invitrogen Corp., Carlsbad, CA, USA). The cultures were incubated in humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37° C.

Furthermore, two oligonucleotides (antisense and sense) containing phosphorothioate backbone were treated into a soft palate cancer (HI) cell line that had exhibited overexpression of the protein. Antisense experiments were performed as described previously.<sup>10</sup> Two oligonucleotides containing phosphorothioate backbones were synthesized as follows: AS, 5'-TCCTGTGCATAGCGTCCGCAGGCC-3' (AS direction of human Skp2 cDNA nucleotides 15 to 40); S, 5'-CCCGGACGCCTGCGATACGTGTCCT-3' (S for AS). The oligonucleotides were delivered into HI cell line directly according to the manufacturer's instructions (Biognostik's Antisense, Germany). The amount of replication was performed in quartet.

Moreover, HI cell line was seeded on 100 mm dish (Falcon, Becton Dickinson Labware, Lincoln Park, NJ, USA) at 2x10<sup>5</sup> cells/well in DMEM containing 10% FCS. After 72 hours, the stucked and floating cells were collected in conical tube (Falcon). Then, the cells were incubated with 5 µl FITC and propidium iodine (PI) in 500 µl binding buffer. Flow cytometry was analysed by a digital flow cytometry system EPICS (Coulter, Miami, FL, USA).

Next, caspase-3 activities were measured using the colorimetric assay kit. 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 HI cell line treated with AS or S were incubated with the substrate (DVED-pNA) in the assay buffer for 2 hours 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.

Furthermore, HI cell line was seeded on 96-well plates (Falcon, NJ, USA) at 5 x 10<sup>3</sup> cells per well in DMEM containing 10% FCS, the day before treatment. Cell line 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-diphenyltetrazolium bromide (Sigma-aldrich) was used.

Therefore, HI cell line ( $5 \times 10^5$  cell/kit) was seeded and placed in the upper compartment (each well was 50  $\mu$ l) and was allowed to migrate through the pores of the membrane into the lower compartment, in which chemotactic agents (medium + p45<sup>Skp2</sup> AS or S) are present. After an appropriate incubation time (24 hours), the membrane between the two compartments is fixed and stained, and the number of cells that have migrated to the lower side of the membrane is determined by light microscope with 40x magnitude.

Statistical differences between the mean for the different groups were evaluated with Stat View 4.5 (Abacus Concepts, Berkeley, CA) using Post-hoc t-test. The significance level was set at 5% for each analysis.

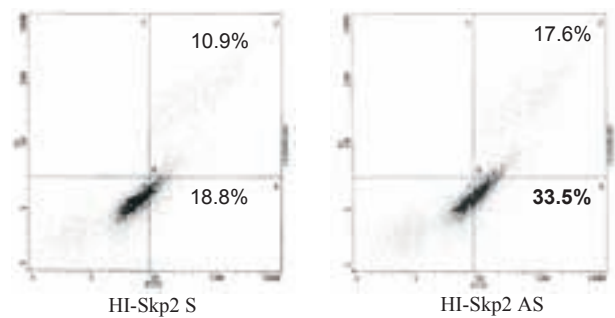
## RESULTS

To determine whether down regulation of p45<sup>Skp2</sup> protein can induce apoptosis, flow cytometry analysis was performed on each transfectant. Flow cytometry analysis demonstrated that high percentage of early apoptosis was detected in HI-Skp2 AS (33.5%) compared with that of HI-Skp2 S (18.8%). However, slightly high percentage of late apoptosis was detected in HI-Skp2 AS (17.6%) (Figure 1). It means HI-Skp2 AS induced apoptosis in both early and late level of apoptosis.

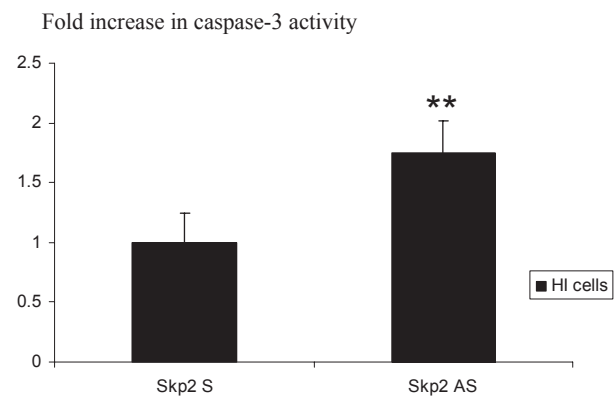
The activity of caspase-3 in HI cells treated with oligonucleotide (AS and S) for 48 hours were examined. As seen in Figure 2, HI-Skp2 AS increased caspase-3 proteolytic activity (> 1.75 fold) compared with that of HI-Skp2 S ( $p < 0.01$ ). It means Skp2 AS was significantly induced the proteolytic activity or apoptosis of HI transfectant cell in external pathway at least 1.75 times compared that of Skp2 S.

Relative cell number was evaluated by comparing the absorbance in each cell using MTT assay at 24 and 48 hours. Relative number of cell HI-Skp2 AS decreased significantly when compared to cell treated by Skp2 S ( $p < 0.05$ ) (Figure 3). It suggests that Skp2 AS was markedly suppressed the HI cell growth effectively compared that of Skp2 S.

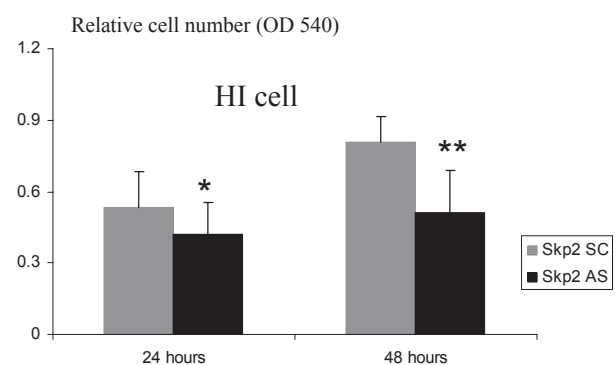
Cell invasion was examined by Boyden chamber kit for 24 hours incubation. As seen in Figure 4, HI cell transfected by Skp2-AS was markedly decreased the cell invasion compared with that of HI-Skp2 S ( $p < 0.05$ ). It means Skp2-AS has strong enough inhibiting the HI cell invasion compared with that of Skp2-S.



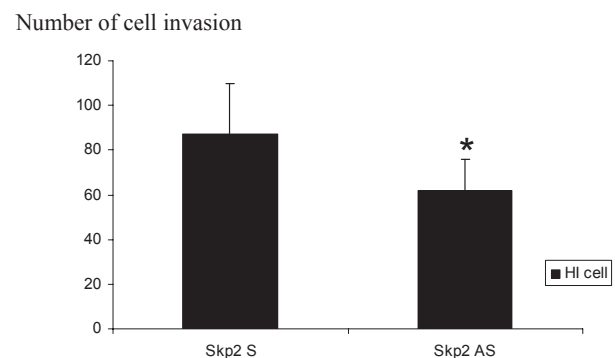
**Figure 1.** Percentages of apoptotic cells in HI-Skp2 oligonucleotide-treated cell.



**Figure 2.** The proteolytic activity of caspase-3 in HI transfectant cells.



**Figure 3.** Growth of HI cell transfectants in vitro at 24 and 48 hours.



**Figure 4.** Suppression of HI-Skp2 cells invasion at 24 hours incubation.

## DISCUSSION

The strategy of therapy with Skp2 AS in human head and neck cancer includes human soft palate cancer becomes the focus of attention in this decade. Antisense oligonucleotides p45<sup>Skp2</sup> contained phosphorothioate backbone was used to knock down protein expression by inhibiting the translation of the mRNA of a desired target gene.<sup>12</sup> Antisense oligonucleotides are synthesized in the hope that they can be used as therapeutic agents-blocking disease processes by suppressing the synthesis of a particular protein. This would be achieved by the binding of the antisense oligonucleotide to the mRNA from which that protein is normally synthesized. Binding of both may physically block the ability of ribosomes to move along the mRNA or simply hasten the rate at which the mRNA is degraded within the cytosol.<sup>14</sup>

In order to be useful in human therapy, antisense oligonucleotides must be able to enter the target cells; avoid digestion by nucleases, and not cause dangerous side-effects. To achieve these goals, 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, antibodies directed against molecules on the surface of the desired target cells. Several commercial factories are presently examining antisense oligonucleotides as weapons against: HIV/AIDS, human cytomegalovirus (HCMV), asthma, several cancers include chronic myelogenous leukemia, and inflammation caused by cell-mediated immune reactions.<sup>15</sup>

In the present study, an antisense strategy to investigate the effect of Skp2 on growth of human soft palate cancer that was overexpressing this gene was employed. Transfection of an antisense oligonucleotide Skp2 into HI cell induced a decrease in growth inhibition followed by invasion cell suppression.

These circumstances, together with observations of cell death in the Skp2-antisense treated cells, prompted us to investigate possible involvement of apoptotic mechanism in the inhibition of cell growth following antisense treatment. An increase in early and late apoptosis percentage and activation of caspase-3 in AS-treated cells strongly suggested that apoptosis had occurred in those cultures. Activation of caspase-3 (an executioner caspase in the apoptosis pathway) leads to the cleavage of poly (ADP-ribose) polymerase (PARP) and DNA fragmentation, indicating that caspase-3 targets cellular proteins for proteolytic cleavage that results in cell death. Caspase-3 can be activated by either an extrinsic apoptosis pathway, by the activation of caspase-8, or an intrinsic apoptosis pathway (via release of cytochrome-c from mitochondria). Activated caspase-8 can directly cleave and activate the executioner caspases, such as caspase-3. Alternatively, it can cleave one of the Bcl-2 family members (such as Bid) to induce the release of mitochondrial cytochrome c, which also leads to activation of caspase-3 via formation of apoptosome (consisting of Apaf-1 and caspase-9).<sup>16</sup>

In the current study, increased caspase-3 activation in AS-treated cells revealed that apoptosis ensured extrinsic pathway. 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 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.<sup>17</sup> As expected from its stronger growth inhibition and apoptosis induction, a significant suppression of tumor growth was detected in HI-Skp2 AS when compared with that of HI-Skp2 S in vivo animal tumor model.

Several investigators have already shown a relationship between expression of Skp2 and apoptosis. S-phase induction by adenovirus-vector mediated expression of Skp2 in quiescent cells was followed by apoptosis.<sup>6</sup> Mouse embryonic fibroblast (MEF) in Skp2-deficient mice showed an increased tendency toward spontaneous apoptosis.<sup>11</sup> Antisense Skp2 and Jab1 induce apoptosis in human oral tongue cancer cells through induction of p27<sup>Kip1</sup> protein.<sup>13</sup> Since components of apoptotic programs represent promising targets for anticancer therapy, down-regulation of Skp2 by the antisense oligonucleotide approach could be a useful apoptosis-modulating strategy for treatment of human head and neck cancers including human soft palate cancers.

In conclusion, antisense oligonucleotide p45<sup>Skp2</sup> has a high antitumor activity in human soft palate cancer, targeting this molecule could represent a promising new therapeutics approach for this type of cancer.

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