

# S-phase kinase-associated protein 2 antisense induces the growth inhibition and apoptosis in a parotid gland cancer cell line

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**ABSTRACT Objective.** To investigate the effect of apoptosis and growth inhibition induced by down-regulation of S-phase kinase-associated protein 2 (Skp2). **Methods.** Two oligonucleotides (antisense and scrambled control) containing a phosphorothioate backbone were used to treat a parotid gland cancer cell line (HSY) that had exhibited overexpression of the protein. **Results.** The number of Skp2 antisense-treated HSY cells was significantly decreased compared to the scrambled control-treated cells ( $P < 0.05$ ). Moreover, Skp2 antisense-treated cells induced apoptosis characterized by an increase in early and late apoptosis, and activation of caspase-3 and -9. The growth of xenograft tumors was also markedly suppressed by Skp2 antisense treatment. **Conclusion.** Skp2 antisense appears to induce growth inhibition and apoptosis in an HSY cell line. Targeting this molecule could represent a promising new therapeutic approach for this type of cancer.

**Key words:** Cell death; Oligonucleotides, antisense; Parotid neoplasms; S-phase kinase-associated proteins

## Introduction

Cancers of the oral cavity present a major health problem, as indicated by their high incidence in many parts of the world. In South-East Asian countries, oral cancers are among the most common and constitute about a third of all cancers <sup>1</sup>. They include parotid gland cancers characterized by a high degree of local invasion and a high rate of metastasis to the cervical lymph nodes. Salivary gland cancer frequently shows local recurrence after initial treatment, probably due to micro-invasion and/or micro-metastasis of tumor cells at the primary site <sup>2</sup>. Conventional treatment of oral cancer involves a combination of surgery, radiotherapy, and chemotherapy. However, overall survival rates have not improved significantly in the last two decades <sup>3</sup>, nor has the prognosis changed during the past 10 years <sup>2</sup>. This

highlights the necessity for continued efforts to improve treatment modalities.

S-phase kinase-associated protein 2 (Skp2), a member of the F-box family, is the substrate-recognition subunit of the SCF<sup>Skp2</sup> ubiquitin ligase complex <sup>4</sup>. The 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>5-7</sup>. The targeted disruption of Skp2 leads to the accumulation of p27 and cell cycle arrest in G<sub>1</sub>. Moreover, Skp2 is also required for ubiquitination of other cell-cycle regulators, including free cyclin E <sup>8</sup>, E2F1 <sup>9</sup>, and hOrclp <sup>10</sup>. Overexpression of Skp2 has been observed in various types of human tumors. Elevated expression of Skp2 indicates poor prognoses for patients with lymphoma <sup>11</sup>, as well as colorectal <sup>12</sup>, gastric <sup>13</sup>, and lung cancers <sup>7</sup>. Therefore, Skp2 knock-out mice grow more slowly and have smaller organs than littermate controls and show increased apoptosis of cells <sup>8</sup>. However, the mechanism of Skp2 overexpression in cancer cells and the nature of its contribution to malignant phenotypes is unclear.

The main purpose of this study was to investigate the effect of apoptosis and growth inhibition induced by down-regulation of Skp2. Two oligonucleotides (antisense [AS] and scrambled control [SC]) containing a

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phosphorothioate backbone were used to treat a parotid gland cancer cell line that had exhibited overexpression of the protein.

## Materials and methods

### Materials

The following materials were used in this study:

1. Dulbecco Modified Eagle Medium (DMEM) and MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) obtained from Sigma-Aldrich, St. Louis, MO, USA;
2. Fetal calf serum (FCS), streptomycin, and penicillin purchased from Moregate BioTech, Bulimba, Australia;
3. Oligonucleotides (AS and SC) containing phosphorothioate backbones obtained from Fasmac Co., Kanagawa, Japan;
4. Fluorescein isothiocyanate (FITC) and propidium iodine (PI) purchased from Annexin V-FITC, BioVision, Inc., Mountain View, CA, USA;
5. Bio-Rad protein assay and polyvinylidene difluoride (PVDF) membrane delivered from Bio-Rad, 2000 Alfred Nobel Drive, Hercules, CA, USA;
6. Rabbit polyclonal antibody against Skp2 and anti- $\alpha$ -tubulin monoclonal antibody purchased from Santa Cruz Biotech, Inc., CA, USA;
7. Monoclonal antibody against p27 Clone 1B4 obtained from Novocastra Lab, Newcastle, UK;
8. Amersham ECL kit delivered from Amersham Pharmacia Biotech, Little Chalfout, Buckinghamshire, UK; and
9. Caspase-3/ CPP32 and -9/Mch6 colorimetric assay kit purchased from BioVision, Inc.

### Cell and cell culture

Derivation of the parotid gland cancer cell line (HSY) has been described<sup>14</sup>. The cell line was cultured in DMEM supplemented with 10% FCS, 100  $\mu$ g/mL streptomycin, and 100 U/mL penicillin. The cultures were incubated in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C.

### Antisense experiments

Antisense experiments were performed as described previously<sup>15</sup>. Two oligonucleotides containing phosphorothioate backbones were synthesized as follows: AS, 5'-TCCT-GTGCATAGCGTCCGCAGGCC-3' (the AS direction of human Skp2 cDNA nucleotide 22-46); SC, 5'-CCCG-GACGCCTGCGATACGTGTCCT-3' (SC for AS)<sup>7,16</sup>.

The oligonucleotides were delivered into the HSY cell line directly, according to the manufacturer's instructions.

### MTT assay

The day before treatment, the HSY cell line was seeded on 96-well plates (Falcon, Becton Dickinson Labware, Lincoln Park [NJ], USA) at 5 x 10<sup>3</sup> cells per well in DMEM containing 10% FCS. The cell line was treated with oligonucleotides at a final concentration of 100  $\mu$ M. After 0, 1, 2 and 3 days, the number of cells was quantitated by an assay making use of MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich)<sup>17</sup>.

### Flow cytometry

The cell line was seeded on a 100 mm dish (Falcon, Becton Dickinson Labware, Lincoln Park [NJ], USA) at 2 x 10<sup>5</sup> cells/well in DMEM containing 10% FCS. After 72 hours, the stuck and floating cells were collected in a conical tube (Falcon). Then, the residual cells were incubated with 5  $\mu$ L FITC and PI in 500  $\mu$ L binding buffer. Flow cytometry analysis was done by a digital flow cytometry system EPICS (Coulter, Miami [FL], USA).

### Western blotting

The cell line was treated with oligonucleotides for 72 hours. Cell lysates were prepared from the treatments as follows: cells were cultured to subconfluence, washed with 100 mM phosphate-buffered saline 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<sub>2</sub>, 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 the Bio-Rad protein assay. Protein samples (50  $\mu$ g) were electrophoresed on SDS-polyacrylamide gel. Proteins from the gels were transferred to a PVDF membrane. The membrane was incubated with a 1:500 dilution of rabbit polyclonal antibody against Skp2 or monoclonal antibody against p27 (Clone 1B4), as the primary antibody using an Amersham ECL kit. Also, anti- $\alpha$ -tubulin monoclonal antibody was used for normalization of the western blot analysis<sup>18</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 was based on the addition of a caspase-specific peptide conjugated to a color reporter molecule p-nitroanilide (pNA). The cleavage of the peptide by caspase releases the chromophore pNA, which is quantified spectrophotometrically at 405 nm. Briefly, equal amounts of cell extracts prepared from HSY cell line treated with AS, SC, or untreated controls (UCs) were incubated with the substrate (DVED-pNA and LEHD-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.

### In-vivo tumorigenesis

The tumorigenicity of tumor cells was examined in the nude mouse with the Balb/cA Jcl-nu genetic background (CLEA Japan, Inc., Tokyo, Japan). Cells ( $1 \times 10^6$ ) of the HSY cell line were suspended in 0.1 mL of serum-free medium and injected into the subcutaneous tissue of 5-week-old nude mice using a 27-gauge needle. Formation of a tumor nodule 10-15 mm in diameter was observed about 10 days after cell inoculation. The examination was started on day 11 after the tumor formed. Mice were randomized into groups and treated with Skp2 SC, Skp2 AS (100  $\mu$ M/day delivered intratumorally on days 0, 3 and 6) and UC cell abstracts. The tumor size was calculated ( $0.4 \times \text{length} \times \text{width}^2$ ) at 3-day intervals. The mice were sacrificed 15 days after study initiation<sup>17</sup>.

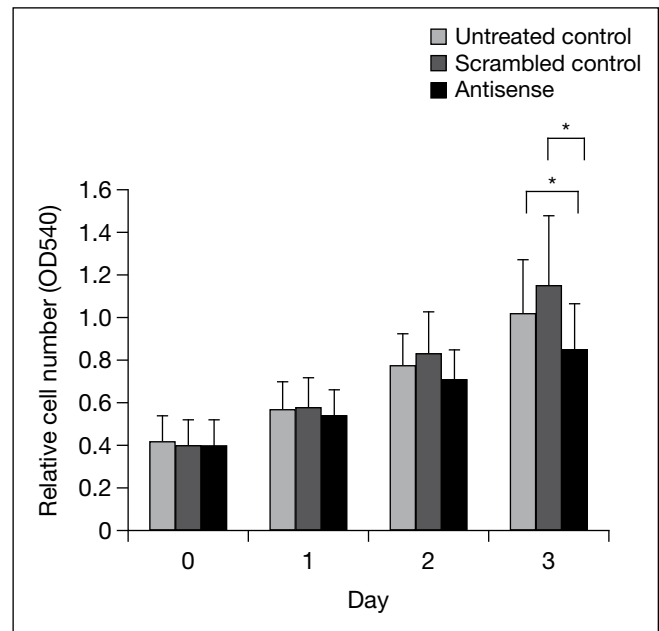
### Statistical analysis

Statistical differences between the means for the different groups were evaluated with Stat View 4.5 (Abacus Concepts, Berkeley [CA], USA) using one-way analysis of variance (ANOVA) and *t* tests. The significance level was set at 0.05 for each analysis.

## Results

### Growth of cells in vitro

In this examination, the effect of oligonucleotides AS or SC at a final concentration of 100  $\mu$ M on in-vitro cell growth of HSY cells was evaluated. Relative cell numbers were evaluated by comparing the absorbance in each cell on days 0, 1, 2 and 3. No significant difference in cell numbers was noted on days 1 and 2. Antisense-treated cells did not suppress the cell growth of HSY cells on day 1. Moreover, AS-treated cells were slightly suppressed



\*  $P < 0.05$  when compared with that of control cells by one-way analysis of variance

**Figure 1** Relative cell number was evaluated by comparing the absorbance in each cell on day 0, 1, 2 and 3 using MTT assay. Values shown are the mean of six determinations; error bars indicate standard deviations

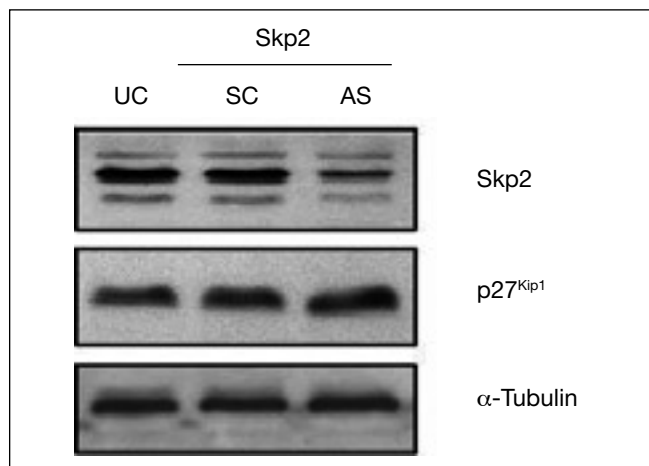
**Table** Mean (standard deviation) percentages of apoptotic cells in S-phase kinase-associated protein 2-treated cell

HSY cell	Untreated control	Scrambled control	Antisense
Early apoptosis	6.4 (1.8)	5.9 (1.4)	12.7 (2.7)
Late apoptosis	8.2 (1.3)	6.1 (1.4)	25.5 (3.2)

on day 2, but not significantly. However, on day 3 the numbers of AS-treated HSY cells were significantly decreased compared to SC-treated cells ( $P < 0.05$ ; one-way ANOVA) [Figure 1].

### Flow cytometry analysis

To determine whether suppression of Skp2 protein can induce apoptosis, flow cytometry analysis was conducted on Skp2 AS-treated cells. This demonstrated early apoptosis in a high percentage following AS treatment ( $12.7\% \pm 2.7\%$ ) in contrast to SC treatment ( $5.9\% \pm 1.4\%$ ). Late apoptosis was also detected in more AS-treated cells ( $25.5\% \pm 3.2\%$ ) than SC-treated cells ( $6.1\% \pm 1.4\%$ ). These results suggested that Skp2 AS had induced apoptosis in HSY cells (Table).



**Figure 2** Expression of S-phase kinase-associated protein 2 (Skp2) and  $\alpha$ -tubulin protein determined by western blotting. The HSY cell line was treated with 100  $\mu$ M antisense (AS), scrambled control (SC), or untreated control (UC) cell abstracts for 72 hours

### Reduction of S-phase kinase-associated protein 2

To examine the expression pattern of Skp2, p27<sup>Kip1</sup>, and  $\alpha$ -tubulin in HSY AS- or SC-treated cells, the protein levels of Skp2, p27<sup>Kip1</sup>, and  $\alpha$ -tubulin were examined by western blot analysis. Accordingly, SC treatment induced an increase of Skp2 protein, whereas AS treatment induced a decrease (<2.1-fold) even in comparison to UC cells. Moreover, up-regulation of p27<sup>Kip1</sup> protein was shown in AS-treated cells (>1.6-fold) as compared to the UC or SC cells. The expression of  $\alpha$ -tubulin as an internal control was approximately the same in all of the

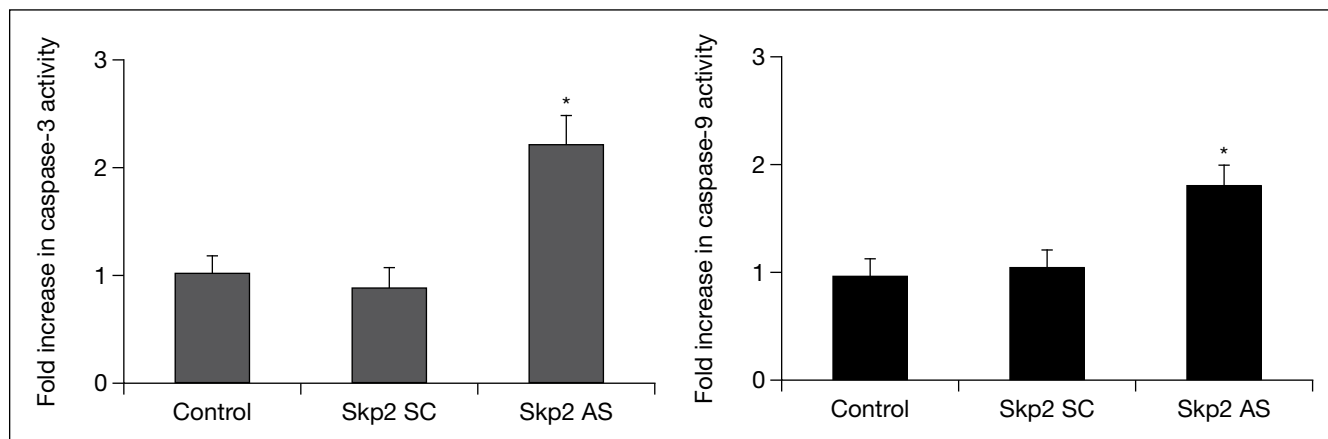
transfected cells (Figure 2).

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

To investigate whether down-regulation of Skp2 protein can induce apoptosis, a colorimetric assay was conducted. Examination of caspase-3 and -9 activity was measured using the colorimetric assay kit according to the manufacturer's directions. The activity of caspase-3 and -9 in HSY cells treated with or without oligonucleotides (AS or SC) for 72 hours was investigated. As seen in Figure 3, HSY cells treated with Skp2 AS increased the proteolytic activity following both caspase-3 and -9 (2.1-fold and 1.8-fold increase, respectively). These results suggested that Skp2 AS induced apoptosis through extrinsic (receptor-mediated apoptosis) and intrinsic (chemical-mediated apoptosis) pathways.

### In-vivo tumorigenicity

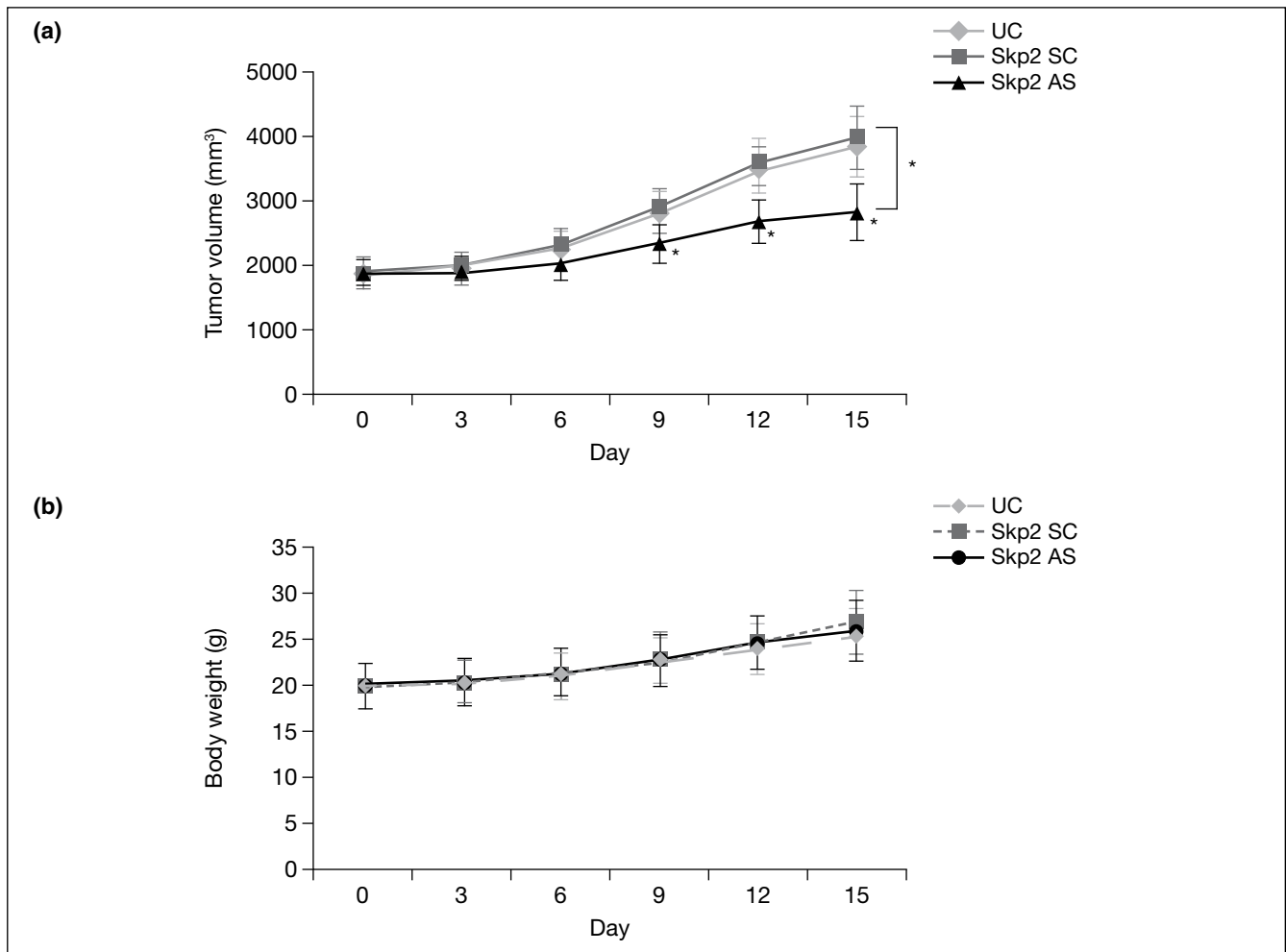
To determine whether suppression of Skp2 protein can inhibit tumor volume, an in-vivo tumorigenicity assay was carried out by examining tumor cells in the nude mouse with a Balb/cA Jcl-nu genetic background. Mice were treated with Skp2 SC, Skp2 AS (100  $\mu$ M/day intratumorally on days 0, 3, and 6), and compared to the UC group. As shown in Figure 4a, the tumor volume was significantly decreased by Skp2 AS treatment when compared to that after Skp2 SC or UC treatment groups ( $P < 0.05$ ). Interestingly, during the experimental period, there was no loss of body weight in either treatment group (Figure 4b), suggesting that the body was not influenced by such treatment.



\*  $P < 0.05$

**Figure 3** The proteolytic activity of caspase-3 and -9 in HSY cell line. Cells were treated with (antisense [AS]) or without (scrambled control [SC]) oligonucleotides

Values are shown as means; error bars indicate standard deviations



\* P<0.05

**Figure 4** Mean (standard deviation shown in error bars) change in (a) tumor volume and (b) body weight in groups of five mice undergoing different treatments (S-phase kinase-associated protein 2 [Skp2] scrambled control [SC], Skp2 antisense [AS], and untreated control [UC] cells)

## Discussion

The orderly transit of cells through the 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 contribute to cancer. 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 regulators (such as the CDK inhibitor p27<sup>Kip1</sup>, for ubiquitin-mediated degradation). A number of studies have found low levels of p27<sup>Kip1</sup> protein in various types of tumors and have associated this decrease with high aggressiveness and poor prognosis<sup>19,20</sup>. High levels of Skp2 expression, and its inverse correlation with p27<sup>Kip1</sup> levels, have been observed in lymphomas<sup>11</sup>, oral cavity squamous cell carcinomas<sup>21,22</sup>, and gastric carcinomas<sup>13</sup>.

In the present study, the AS strategy was employed to investigate the effect of Skp2 on growth of a parotid gland cancer cell line that was overexpressing this protein. Treatment with AS introduced into cultured HSY cells induced the growth inhibitory effect, in contrast to SC or UC treatment. These results clearly showed that growth was inhibited by the AS effect and not by non-specific effects such as oligonucleotide toxicity. Lately, Schlingensiepen and Schlingensiepen<sup>23</sup> 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. It has been reported that the mechanism of action of AS oligonucleotide can be discerned through the RNase H-dependent oligonucleotides<sup>24</sup>. 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. In contrast to the steric-blocker oligonucleotides, RNase H-dependent oligonucleotides can inhibit protein expression when targeted to virtually any region of the mRNA<sup>24</sup>. Thus, whereas most steric-blocker oligonucleotides are efficient only when targeted to the 5'- or AUG initiation codon region, phosphorothioate oligonucleotides can inhibit protein expression when targeted to widely separated areas in the coding region<sup>25</sup>. As expected from the growth inhibitory effect, a decrease in Skp2 protein in AS-treated cells was followed by an increase in p27<sup>Kip1</sup> protein. These results suggest that alterations of Skp2 and p27<sup>Kip1</sup> expression levels may be closely associated with malignancy progression<sup>14</sup>. It has been reported that the molecular mechanisms of Skp2 and p27<sup>Kip1</sup> have an inverse correlation<sup>24</sup>. Up-regulation of Skp2 or functional loss of p27<sup>Kip1</sup> has been implicated in carcinogenesis and cancer progression<sup>14</sup>. Several investigators reported that degradation of p27<sup>Kip1</sup> can be promoted by phosphorylation of Skp2 and Thr-187<sup>14,26</sup>. Moreover, an increase in early and late apoptosis percentages and activation of caspase-3 and -9 in AS-treated cells strongly suggest that apoptosis occurred in those cultures. Similar results were reported by Harada *et al.*,<sup>27</sup> who suggested that in oral cancer, induction of apoptosis can be caused by down-regulation of Skp2. Therefore, Azuma *et al.*<sup>28</sup> reported that activation of caspase-3 (an executioner caspase in the apoptosis pathway) leads to the cleavage of 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>27</sup>. In the current study, increased caspase-3 and -9 activation in AS-treated cells revealed that apoptosis ensued extrinsic and intrinsic pathways. Furthermore, a stronger anti-tumor effect of Skp2 AS was reconfirmed by the in-vivo tumorigenicity assay in nude mice. As expected from its stronger growth

inhibition and apoptosis induction properties, in the in-vivo animal tumor model, significant suppression of tumor growth was detected following Skp2 AS treatment rather than after SC treatment. Interestingly, AS treatment of cells did not affect the body weight of the nude mice, suggesting that AS treatment may be safe.

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>7</sup>. Mice embryonic fibroblasts in Skp2-deficient mice showed an increased tendency toward spontaneous apoptosis<sup>8</sup>. Also, Skp2-transfected gastric cancer cells showed resistance to induction of apoptosis by actinomycin D<sup>13</sup>. However, the actual role of Skp2 in apoptosis remains unclear. In the present study, Skp2 AS led to apoptosis. This result may be compatible with the overexpression of p27<sup>Kip1</sup> that is able to induce apoptosis in several cancer cell lines<sup>15,19,21</sup>, as expression of Skp2 is inversely correlated with that of p27<sup>Kip1</sup><sup>29</sup>.

In conclusion, suppression of Skp2 regulation by AS treatment could induce apoptosis and growth inhibition in a parotid gland cancer cell line. Furthermore, since components of apoptotic programs represent promising targets for anticancer therapy, down-regulation of Skp2 by the AS approach could be a useful apoptosis-modulating strategy for the treatment of several cancers.

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## Answers to CPD Programme Hong Kong Dental Journal June 2008 issue

Hong Kong Dent J 2008;5:11-8

Implant reconstructions in periodontally susceptible patients

1. b
2. d
3. a
4. d