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Down Regulation of Jun Activation Domain-Binding Protein-1 Induces Cell Cycle Arrest and Apoptosis in a Human Parotid Gland Cancer Cell

Supriatno

Department of Oral Medicine, Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta, Indonesia

Abstract: ***Jun activation domain-binding protein-1 (Jab1) is a multifunctional protein that regulates cell proliferation, apoptosis and oncogenesis by interacting with and subsequently degrading a large number of proteins. Overexpression of Jab1 indicated the aggressiveness and a poor prognosis of some type of cancers. The antisense (AS) effect was confirmed in a cell line of adenocarcinoma parotid gland (HSY) that also exhibited overexpression of the protein. In the present study, the mechanism responsible for AS mediated cell cycles arrest and apoptosis of HSY cells was examined. The cell cycle arrest of Jab1 AS treated HSY cell was significantly increased when compared with that of control (SC) cells (P<0.05). Jab1 AS-treated cell induced apoptosis characterized by an increase in the activation of caspase-3 and -9. Furthermore, up-regulation of p27Kip1 was detected in Jab1 AS treated cells. These results suggest that Jab1 AS appears to induce cell cycle arrest and apoptosis in a HSY cell line, targeting this molecule could represent a promising new therapeutic approach for this type of cancer.***

Keywords: Jab1 AS, apoptosis, parotid gland cancer, cell cycle arrest, p27Kip1

1. Introduction

Salivary gland tumours are estimated to represent approximately 3% of all head and neck tumours. About 70–80% of these neoplasms occur to the major salivary glands, with the parotid gland being the most commonly affected site.[1] Salivary gland cancer frequently shows local recurrence after initial treatment, probably due to micro invasion and/or micro metastasis of the tumor cells at the primary site.[2] Treatment of oral cancer is conventionally a combination of surgery, radiotherapy, and chemotherapy. However, the overall survival rates have not improved significantly on the last two decades. [3)] Also, the prognosis has not changed into the past 10 years. [2)] This highlights the necessity for continued efforts to improve the treatment modalities.

Jun activation domain-binding protein-1 (Jab1) is a subunit (CSN5) of the constitutive photomorphogenesis 9 signalosome (CSN), which is an evolutionarily conserved and multifunctional protein that involves in controlling cellular proliferation and apoptosis, affecting a series of pathways, as well as regulating genomic instability and DNA damage and repair.[4] Jab1 was identified as a coactivator of the gene regulatory activator protein (AP-1).[5] Jab1 is a nuclear export protein that targets $p27^{Kip1}$ for transportation from the nucleus to the cytoplasm and promotes its subsequent degradation.[6] Jab1 can interact with a number of proteins and regulates their function, and is involved in different signal transduction pathways, including degradation of target proteins by regulating gene transcription and cell cycle through phosphorylation.[7] It was reported Jab1 regulates cell proliferation through p27.[8] These findings indicate that Jab1 may play a significant role in oncogenesis. Furthermore, the expression of Jab1 is inversely correlated with $p27^{Kip1}$ protein expression, and is significantly associated with adverse clinicopathological characteristics. Recent studies reported that the increase of the Jab1 expression level was correlated with a decrease of $p27^{Kip1}$ levels and poor prognosis in ovarian [9], pancreatic,[10] and colon cancer.[11]

The main purpose of this study was to investigate the effect of apoptosis and the cell cycle arrest induced by down-regulation of Jab1. Two oligonucleotides (AS and SC) containing phosphorothioate backbone were treated into a parotid gland cancer (HSY) cell line that had exhibited overexpression of the protein.

2. Materials and Methods

2.1 Cell and Cell Culture

Raji cell line (ATCC CCL-86 B-lymphocyte, USA) was obtained from Department of Paracytology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. The 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 a humidified atmosphere of 95% air and 5% $C0_2$ at 37^0C.

2.2 Antisense experiments

Antisense experiments were performed as described previously.[12] Briefly, two oligonucleotides containing phosphorothioate backbones were synthesized (Fasmac , Japan) as follows: AS, 5'-CGGACGCCGCCATCGCCG AGGAAG-3' (the antisense direction of human Jab1 cDNA

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nucleotides 15 to 24), Sense (S), 5'-CCTCTTACCTCAGTTACAATTTATA-3' (the sense direction of human Jab1 cDNA nucleotides 15 to 25). The oligonucleotides were transfected into HSY cells using Tris-EDTA reagen according to the manufacturer's instructions.

2.3 Cell cycle analyses by flow cytometry

The distribution of cells among the different phases of their growth cycles was achieved by using a published method with slight modification.[13] Briefly, after washing the cells for twice with PBS and re-suspending in the hypotonic solution containing 10 mM Tris/HCl, 0.1% Triton X-100, 200 µg/ml RNase and 50 µg/ml propidium iodide, they were incubated at room temperature for 30 min. The stained cells were analyzed by flow cytometry.

2.4 Detection of apoptosis using colorimetric assay

Induction of apoptosis was measured using the colorimetric assay kit (caspase-3 and-9; BioVision Research Product, CA, USA) according to the manufacturer's directions. Briefly, equal amounts of tissue extracts prepared from Raji treated cells were incubated with the substrate (DVED or LEHD-pNA) in the assay buffer for 2 hours at room temperature. Absorbance was measured at 450 nm using a microplate reader (BioRad, USA). Each determination was performed in triplicate.

2.5 Western Blot Analysis

Cell lysates were prepared from the Raji-treated cells in Falcon tissue culture for 48 h. Briefly, samples containing equal amounts of protein (50 µg) were electrophoresis on an SDS-polyacryl-amide gel and transferred to a polyvynilidene fluoride (PVDF) membrane (BioRad, Hercules, CA, USA). The filters were blocked in TBS containing 5% non-fat milk powder at 370C for 1 h, and then incubated with a 1:500 dilution of primary antibodies against the p27 protein (clone 1B4, mouse monoclonal antibody; Novocastra Laboratories, New Castle, UK), 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.

3. Results

3.1 Induction of cell cycle arrest

After treatment of HSY cells with Jab1 AS for 24 hours, the cell population in G1 phase increased steadily from 62.2 to 70.9 % which was accompanied with a slight decrease in S (6.1 %) and G2/M (3.7 %) phases. These results clearly indicated that Jab1 AS induced cell cycle arrest in G1 phase (Table 1).

Table 1: Percentages of cell cycle population in Jab1 AS-treated cell

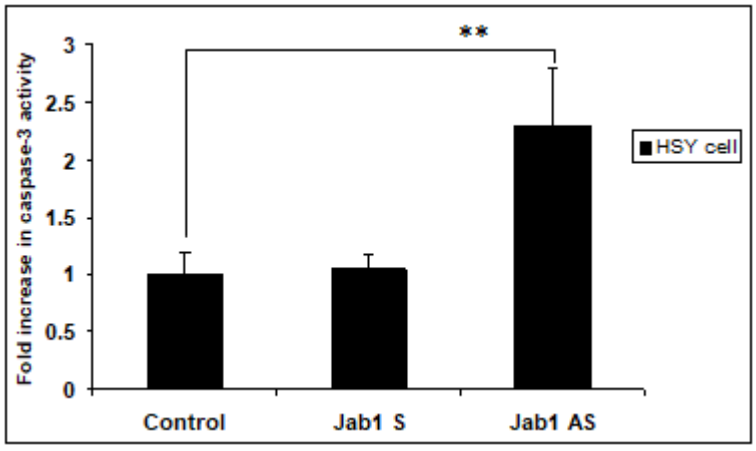
	G1 (%)	S (%)	G2/M (%)
Jab1 control	62.2	9.8	25.2
Jab1 sense (S)	63.7	8.7	26.1
Jab1 antisense (AS)	**70.9**	3.7	21.5

3.2 Jab1AS induces apoptosis by activation of caspases

The activity of caspase-3 and -9 in HSY cells treated with oligonucleotide AS or S for 48 hours was examined. As seen in Figure 1, Jab1 AS was increased the caspase-3 activity at 2.3 fold, whereas the caspase-9 activity in 1.6 fold. It was suggested that caspase-3 and -9 mediates-AS induced apoptosis.

3.3 Reduction of Jab1 protein

Western blot analysis demonstrated that a sense induce an increase in Jab1 protein. However, an antisense oligonucleotide (AS) induced a decrease in Jab1 protein that become most notable 48 hours after transfection of AS into HSY cells. Furthermore, up-regulation of p27 protein was detected in HSY-Jab1 AS cells compared with that of HSY-Jab1 S and control. The expression of α-tubulin as an internal control was approximately the same in all of the treated cells (Figure 2).



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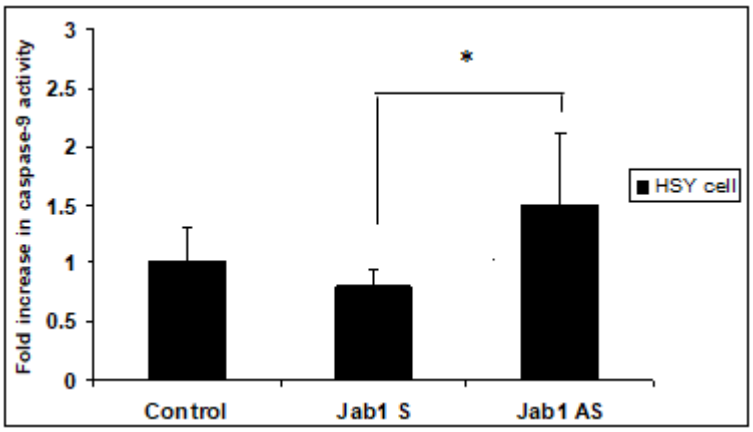

Figure 1: The activity of caspase-3 and caspase-9 in HSY cell treated with Jab1 AS or S

4. Discussion

The orderly transit of cells through the cell cycle requires a delicate balance between positive and negative regulatory factors. Any alteration in this condition can result in abnormal cell proliferation, which may contribute to cancer. Through detailed knowledge of oncogenic signal transduction pathways, targeted therapies have provided exciting advancements in the treatment of cancer where standard chemotherapy alone has failed. [14] During this period, increased growth, invasion and metastasis suppression and apoptosis induction of cells through targeted therapies have become the attention center of researchers in the field of oral cancer. [15]

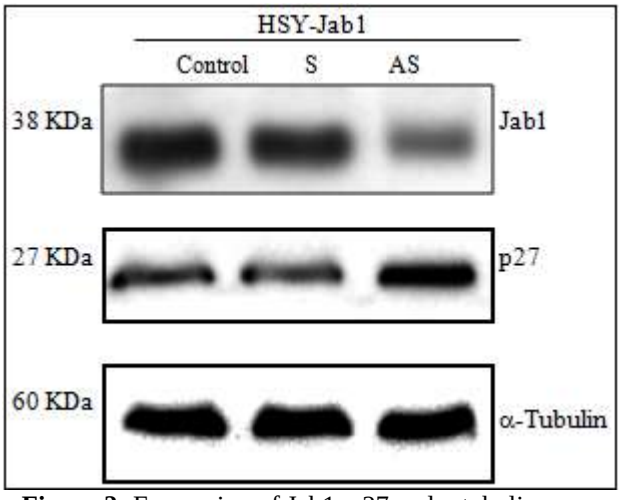

Figure 2: Expression of Jab1, p27 and a-tubulin were determined by Western blotting analysis.

Jab1/CSN5 promotes cell proliferation by interacting directly with $p27^{Kip1}$ (p27) and induces nuclear export and subsequent p27-degradation. p27 is a critical component of the cell-cycle machinery.[16] As a negative regulator of the cell cycle and an inhibitor of cyclin E-Cdk2 and cyclin D-cdk4, p27 plays a pivotal role in controlling cell proliferation and therefore the cell's entry into S phase and exit from G1 phase during development and tumorigenesis.[17-18] Increasing the level of Jab1 causes an elevating breakdown of p27 and indicated that Jab1 controls the activity of p27 by facilitating its degradation. This finding suggested that Jab1 can act as a positive regulator of important cell cycle control by targeting them for degradation.[19]

In the present study, an antisense strategy to investigate the effect of Jab1 on cell cycle arrest and apoptosis of human salivary gland cancer that were overexpressing this gene was examined. Transfection of Jab1 antisense oligonucleotide into cultured HSY cells induced cell cycle arrest in G1 phase (Table 1) and decreased Jab1 protein (Fig. 2). These circumstances, together with observation of cell death in the Jab1 AS treated cells, prompted me to investigate the possible involvement of apoptotic mechanism in the suppression of cell cycle arrest following AS treatment. An increase in activation of apoptosis characterized by induction of caspase-3 and -9 activities (Fig. 1) in the AS-treated cells strongly suggested that apoptosis had occurred in those cultures. In addition, Jab1 AS may induce apoptosis through the external and internal mechanism pathways. An antitumor effect of Jab1 AS was reconfirmed by an up-regulation of p27 and down-regulation of Jab1 through Western blot analysis. As expected from its potency in cell cycle arrest and apoptosis induction, a marked suppression of Jab1 protein was detected in HSY-Jab1 AS when compared with that of HSY-Jab1 S and control (Fig. 2).

Several investigators had already detected a relationship between Jab1 and apoptosis or cell cycle arrest in their experiments. Jab1/CSN5 is a new player in cell cycle control and cancer, resulting in the tumorigenic process.[14] Jab1/CSN5 controls multiple events in the mammalian cell cycle.[21] Jab1 is a specificity factor for E2F1-induced apoptosis, depleting cells of Jab1 by short hairpin RNA (shRNA) should impair the ability of E2F1 to induce apoptosis. These results suggested that Jab1 may act as a key molecule in cell death by association with impair the ability of E2F1. [20] However, the actual role of Jab1 in apoptosis remains unclear. In our study detected that down regulation of Jab1 led to apoptosis and cell cycle arrest followed by up-regulation of p27 and down-regulation of Jab1. This result may be compatible with the observation that overexpression of p27 was able to induce

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apoptosis in several cancer cell lines, because expression of Jab1 is inversely correlated with that of p27 in oral cancer. [22]

Since the component of apoptotic programs represents promising targets for anticancer therapy, down-regulation of Jab1 by the antisense approach could be a useful apoptosis-modulating strategy for treatment of oral cancer. Further investigation of Jab1/CSN5 as a therapeutic target may lead to the development of a powerful cancer therapy for use in a wide range of tumors.

In conclusion, Jab1 AS appears to induce cell cycle arrest and apoptosis in a HSY cell line, targeting this molecule could represent a promising new therapeutic approach for this type of cancer.

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Author Profile

Dr. Supriatno., M,Health., MDSc., Ph.D, Bandung, May 13, 1967. Author can be contacted at Oral Medicine Department, Fakulty of Dentistry, Univeristas Gadjah Mada, Jl. Denta 1, Sekip, Utara, Yogyakarta, 55281. Indonesia

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