Jun Activation Domain-binding Protein 1 Antisense ($p27^{Kip1}$) Induces Apoptosis of an Oral Tongue Cancer Cell

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ABSTRACT

The Jun activation domain-binding protein 1 (Jab1), aside from being an activator protein 1 coactivator, is involved in degradation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$, a dosage-dependent tumor suppressor protein. The antisense effect was confirmed in an oral tongue cancer cell line Supri's clone-1 (Sp-C1). In this study, the antisense-mediated apoptosis of Sp-C1 derived cultures was analized. Jab1-antisense (Jab1-AS) treatment induced apoptosis characterized by an increase in the early apoptosis (33.5%) and late apoptosis (17.6%). Also, the proteolitic activation of caspase-3 and caspase-9 was increased. These data suggest that Jab1 AS could induce apoptosis in oral tongue cancer cell (Sp-C1). Targeting this molecule could represent a promising new therapeutic approach for this type of cancer.

***Key words**: Jab1, apoptosis, antisense, oral tongue cancer (Sp-C1).*

ABSTRAK

Jun activation domain-binding protein (Jab1) berperan sebagai koaktivator dari aktivator protein 1 yang terlibat dalam degradasi *cyclin-dependent kinase inhibitor* $p27^{Kip1}$. Efek Jab1 antisense (Jab1-AS) diujikan pada sel kanker lidah manusia *Supri's-Clone 1* (Sp-C1). Tujuan penelitian ini adalah menganalisis hambatan sel SP-C1 dengan perlakuan Jab1-AS. Hasil penelitian menunjukkan bahwa Jab1-AS meningkatkan apoptosis yang ditandai dengan meningkatnya fase awal apoptosis (33,5%) dan fase lambat apoptosis (17,6%). Selain data tersebut, diketahui peningkatan aktivitas proteolitik caspase-3 dan caspase-9 pada sel yang diperlakukan dengan Jab1 AS. Kesimpulannya, Jab1 AS dapat meningkatkan apoptosis sel kanker lidah manusia (Sp-C1). Target pada molekul Jab1 dapat memberikan harapan baru sebagai pendekatan terapeutik untuk jenis kanker tersebut.

Kata kunci: Jab1, apoptosis, antisense, kanker lidah manusia (Sp-C1).

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INTRODUCTION

Human oral tongue squamous cell carcinoma (OTSCC) accounts for the majority of malignancies that arise in head and neck region.[1] OTSCC is characterized by a high degree of local invasion and a high rate of metastases to the cervical lymph nodes. Moreover, human oral tongue cancer cells frequenly shows local recurrence after initial treatment, probably due to microinvasion and/or micrometastasis of the tumor cells at the primary site. Therefore, the ratio of mortality/incidence in 1980 and 1990 was 0.48 and 0.47, respectively, and the prognosis has not changed during the past 10 years.[2,3]

The human Jab1 was originally identified as a coactivator of the gene-regulatory AP-1 proteins (Jun/Fos proto-oncogenes) involved in the control of cell proliferation.[4] Jab1 is also known as COP9 singnalosome subunit 5 (CSN5), which is a component of the COP9 singnalosome regulatory complex.[5,6] Recently, it was demonstrated that Jab1 can specifically interact with the cyclin-dependent kinase (CDK) inhibitor protein $p27^{Kip1}$.[7] Transient coexpression of Jab1 with $p27^{Kip1}$ accelerated the degradation of $p27^{Kip1}$ in mammalian cells by translating $p27^{Kip1}$ from the nucleus to the cytosol, in which degradation could occur.[7] $p27^{Kip1}$ is a universal CDK inhibitor that directly inhibits the enzymatic activity of cyclin-CDK complexes, resulting in cell cycle arrest at G_1.[8] Recently, decreased expression of $p27^{Kip1}$ has been frequently detected in human cancer,[9-15] including head and neck carcinoma.[16-20] These studies indicated that $p27^{Kip1}$ protein level may be associated with the development of human cancers and appear as an important marker of cancer progression. In the latter study, Jab1 overexpression was inversely associated with $p27^{Kip1}$ levels .[21-24] However, to the best of our knowledge, the status of Jab1 expression in human oral tongue cancer has not been examined.

In the present study, the mechanism of the observed growth inhibition induced by Jab1 AS was explored. Antisense oligonucleotides into human oral tongue cancer cell lines (Sp-C1) that had exhibited amplification and consequent overexpression of the gene was transfected.

MATERIALS AND METHODS

Cell and Cell Culture

Oral tongue squamous cell carcinoma cell lines (Sp-C1) was used in this study. Sp-C1 cell line was establised in our laboratory.[25-28] The cell line was grown in Dulbecco's modified Eagle medium (DMEM, Sigma, St Louis, MO, USA) supplemented with 10% fetal calf serum (FCS, Moregate BioTech, Bulimba, Australia), and 100 μg/ml streptomycin, 100 units/ml penicillin (Invitrogen Corp., Carlsbad, CA, USA). Sp-C1 cell cultures were incubated in humidified atmosphere of 95% air and 5% Co_2 at 37°C.

Antisense Experiments

Antisense experiments were performed as described previously.[24] Briefly, two oligonucleotides containing phosphorothioate backbones were synthesized (Fasmac , Japan) as follows: AS, 5'-CGGACGCCGCCATCGCCGAGGAAG-3' (the antisense direction of human Jab1 cDNA nucleotides 15 to 24), Sense (S), 5'-CCTCTTACCTC AGTTACAATTTATA-3' (the sense direction of human Jab1 cDNA nucleotides 15 to 25). The oligonucleotides were transfected into Sp-C1 cells using Tris-EDTA reagent according to the manufacturer's instructions.

Flow Cytometry

Cells were seeded on 100 mm dish (Falcon, USA) at $2x10^5$ cells/well in DMEM containing 10% FCS. After 72 hours, the sticked and floating cells were collected in conical tube (Falcon, USA). Then, the cells were incubated with 5μl FITC and propidium iodine (PI) in 500 μl binding buffer (Annexin V-FITC, Bio Vision Inc, CA). Flow cytometry was analysed by a digital flow cytometry system EPICS (Coulter, Miami, FL, USA).

Caspase-3 and –9 Activity

Caspase-3 and -9 activity were measured using the colorimetric assay kit (BioVision, caspase-3/CPP32 and caspase-9/Mch6 colorimetric assay kit, CA, USA) 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. The cleavage of the peptide by caspase releases the chromophore p-NA, which is quantitated spectrophotometrically at 405 nm.[30] Briefly, equal amounts of cell extracts prepared from Sp-C1 cells treated with AS or S were incubated with the substrate (DVED-pNA and LEHD-pNA) in the assay buffer for 2 hours at room temperature. Absorbance was measured at 405 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA). Each determination was performed in triplicate.

Statistical Analysis

Statistical differences between the means for the different groups were evaluated with Stat View 4.5 (version 5.0J, SAS Institute Inc, Cary, NC, USA) using one-way ANOVA and *t-test*. The significance level was set at 5% for each analysis.

RESULTS

Flow Cytometry Analysis

To determine whether down regulation of Jab1 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 Sp-C1 Jab1 AS (33.5%) compared with that of Sp-C1 Jab1 S (18.8%). Furthermore, percentage of late apoptosis was found in Sp-C1 Jab1 AS (17.6%) compared with of Sp-C1 Jab1 S (17.6%) (Fig.1).

Kip 1 Induces Apoptosis by Activation of Caspases

The activity of caspase-3 and -9 in Sp-C1 cancer cells treated with or without oligonucleotide (AS and S) for 72 h was examined. As seen in Fig. 2, Sp-C1 Jab1 AS showed increased caspase-3 (2.4 fold) and -9 (1.9 fold) proteolytic activity compared with the respective neo-transfectant or

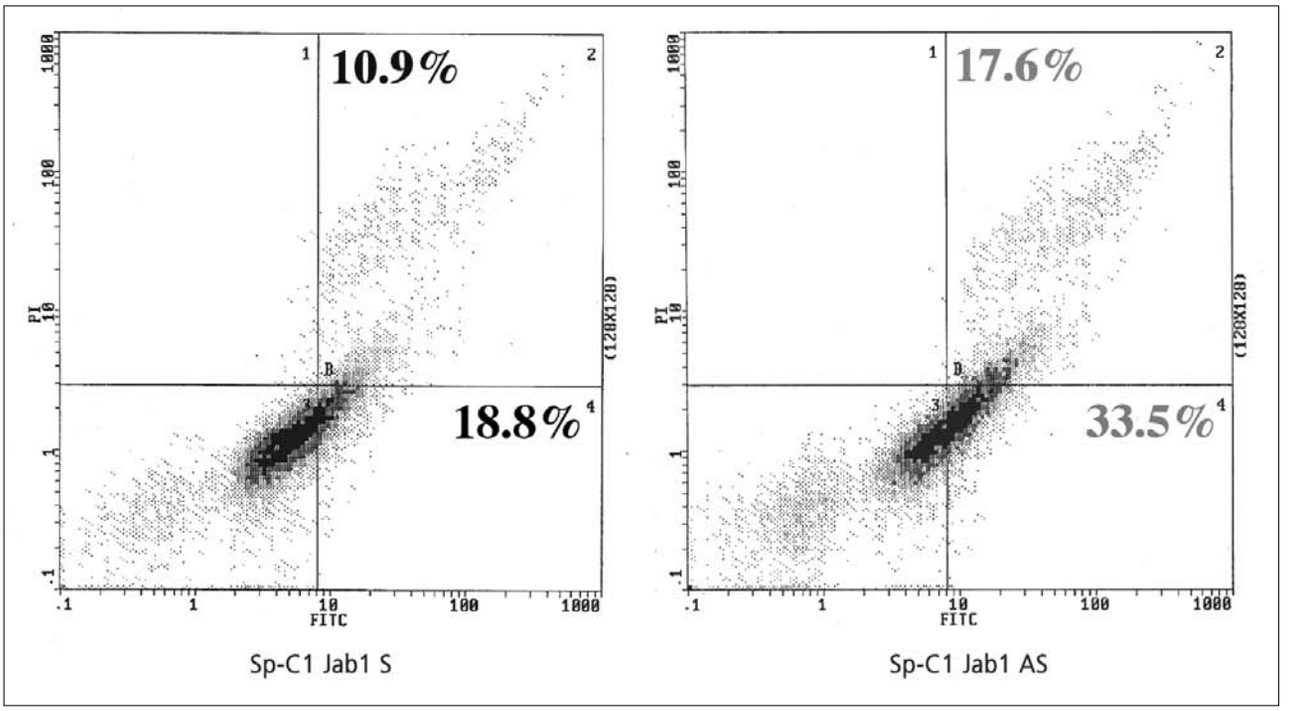

Figure 1: Percentages of apoptosis cells in oligonucleotide-treated Sp-C1 cells

sense treated cell. These data suggest that Sp-C1 Jab1 AS induced apoptosis through external and internal pathways.

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. Jab1 was initially described as a coactivator of AP1 transcription factor and is a subunit of a large protein complex (called the COP9 signalosome). Recent study found that an increasing level of Jab1 causes an increasing breakdown of $p27^{Kip1}$ and indicated that Jab1 controls the activity of $p27^{Kip1}$ by facilitating its degradation. This finding suggested that Jab1 can act as a negative regulator of important cell cycle control proteins by targeting them for degradation.[7]

The study of Claret *et al*. by immunofluorescence analysis indicated that Jab1 is a nuclear protein.[4] Through selective interaction with the Jun proteins, Jab1 can increase the specificity of target-gene activation by this large family of related transcription factors. However, to date, the detailed biological functions of Jab1 in mammalian cells have not been identified.

In the present study, an antisense strategy to investigate the effect of Jab1 on growth of head and neck cancer cells that were overexpressing this gene was employed. Transfection of an antisense oligonucleotide

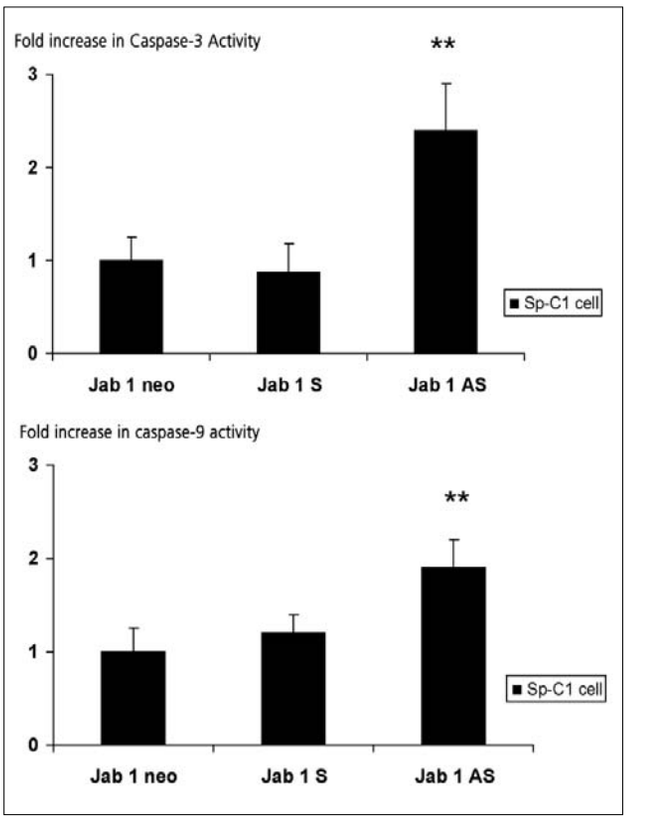

Figure 2: The activity of caspase-3 and -9 in Sp-C1 cell. Caspases proteolytic activity was assessed using a colorimetric assay.

(AS) into cultured Sp-C1 cells induced early and late apoptosis (Fig.1), followed by increasing proteolytic activity of caspase-3 (2.4 fold) and -9 (1.9 fold) (Fig. 2). These circumstances, together with observations of cell death in the Jab1-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 percentation and activation of caspase-3 and –9 in AS-treated cells strongly suggested that apoptosis had occurred in those cultures.

Several investigators had already detected a relationship between Jab1 and apoptosis in their experiments. Jab1 -/- embryonic cells, which lacked other CSN components, expressed higher levels of p27, p53 and cyclin E, resulting in impaired proliferation and accelerated apoptosis.[31] Mutant Jab1 down-regulates the UPR signaling pathway through tight binding with IRE1α These result suggested that Jab1 may act as a key molecule in selecting the UPR or cell death by association and dissociation with IRE1α.[32] However, the actual role of Jab1 in apoptosis remains unclear. In our study found that down regulation of Jab1 led to apoptosis. This result may be compatible with observation that overexpression of p27^{Kip1} was able to induce apoptosis in several cancer cell lines, because expression of Jab1 is inversely correlated with that of p27^{Kip1} in oral squamous cell carcinomas.[33,34]

Since components of apoptotic programs represent promising targets for anticancer therapy, down-regulation of Jab1 by the antisense approach could be a useful apoptosis-modulating strategy for treatment of head and neck cancers. Further studies are needed to clarify the apoptotic mechanism revealed here, and to determine whether Jab1 down-regulation also induces apoptosis in cells derived from tumors of other types.

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REFERENCES

1. Hitt R, Jimeno A, Millan JM, Castellano D, Cortes-Funes H. Phase II trial of dose-dense paclitaxel, cisplatin, 5-fluorouracil, and leucovorin with filgrastim support in patients with squamous cell carcinoma of the head and neck. *Cancer* 2004; 101:768-87.
2. Pisani P, Parkin DM, Bray F, Ferlay J. Estimates of the worldwide mortality from 25 cancers in 1990. *Int J Cancer* 1999; 83:18-29.
3. Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of 25 major cancers in 1990. *Int J Cancer* 1999; 80:827-41.
4. Claret FX, Hilbi M, Dhut S, Toda T, Karin M. A new group of conserved coactivators that increase the specificity of AP-1 transcription factor. *Nature* (Lond) 1996; 383:453-7.
5. Seeger M, Kraft R, Ferrell K, Bech-Otschir D, Dumdey R, Schade R, *et al*. A novel protein complex involved in signal transduction possessing similarities to 26S proteasome subunit. *FASEB J* 1998; 12:469-78.
6. Chamovitz DA, Segel D. Jab1/CSN5 and the COP9 signalosome. A complex situation. *EMBO Rep* 2001; 2:96-101.
7. Tomoda K, Kubota Y, Kato J. Degradation of the cyclin-dependent kinase inhibitor p27^{Kip1} is instigated by Jab1. *Nature* (Lond) 1999; 398:160-5.
8. Sherr C, Roberts JM. Inhibitors of mammalian G_1 cyclin-dependent kinases. *Genes Dev* 1995; 9:1149-63.
9. Catzavelos C, Bhattacharya N, Ung YC, Wilson JA, Roncari L, Sandhu C, *et al*. Decreased levels of the cell-cycle inhibitor p27^{Kip1} protein: prognostic implications in primary breast cancer. *Nat Med* 1997; 3:227-30.
10. Porter PL, Malone KE, Heagerty PJ, Alexander GM, Gatti LA, Firpo EJ, Daling JR, Roberts JM. Expression of cell-cycle regulators p27^{Kip1} and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. *Nat Med* 1997; 3:222-5.
11. Cordon-Cardo C, Koff A, Drobnjak M, Capodieci P, Osman I, Millard SS, *et al*. Distinct altered patterns of p27^{Kip1} gene expression in benign prostatic hyperplasia and prostatic carcinoma. *J Natl Cancer Inst* (Bethesda) 1998; 90:1284-91.
12. Mori M, Mimori K, Shiraishi T, Tanaka S, Ueo H, Sugimachi K, Akiyoshi T. p27^{Kip1} expression and gastric carcinoma. *Nat Med* 1997; 3:593.
13. Esposito V, Baldi A, De Luca A, Groger AM, Loda M, Giordano GG, *et al*. Prognostic role of the cyclin-dependent kinase inhibitor p27^{Kip1} in non-small cell lung cancer. *Cancer Res* 1997; 57:3381-5.
14. Florenes VA, Maelandsmo GM, Kerbel RS, Slingerland JM, Nesland JM, Holm R. Protein expression of the cell-cycle inhibitor p27^{Kip1} in malignant melanoma: inverse correlation with disease-free survival. *Am J Pathol* 1998; 153:305-12.
15. Loda M, Cukor B, Tam SW, Lavin P, Fiorentino M, Draetta GF, Jessup JM, Pagano M. Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27^{Kip1} in aggressive colorectal carcinomas. *Nat Med* 1997; 3:231-4.
16. Venkatesan TK, Kuropkat C, Caldarelli DD, Panje WR, Hutchinson JC Jr, Chen S, Coon JS. Prognostic significance of p27 expression in carcinoma of the oral cavity and oropharynx. *Laryngoscope* 1999; 109:1329-33.
17. Supriatno, Harada K, Hoque MO, Bando T, Yoshida H, Sato M. Overexpression of p27^{Kip1} induces growth arrest and apoptosis in an oral cancer cell line. *Oral Oncol* 2002; 38:730-6.
18. Supriatno, Harada K, Kawaguchi SI, Onoue T, Yoshida H, Sato M. Characteristics of antitumor activity of mutant type p27^{Kip1} gene in an oral cancer cell line. *Oral Oncol* 2004; 40:679-87.
19. Harada K, Supriatno, Yoshida H, Sato M. Low p27^{Kip1} expression is

associated with poor prognosis in oral squamous cell carcinoma. *Anticancer Res* 2002; 22:2985-90.

20. Supriatno, Harada K, Kawaguchi S, Yoshida H, Sato M. Effect of $p27^{Kip1}$ on the ability of invasion and metastasis of an oral cancer cell line. *Oncol Rep* 2003; 10:527-32.
21. Tomoda K, Kubota Y, Arata Y, Mori S, Maeda M, Tanaka T, Yoshida M, Yoneda-Kato N, Kato JY. The cytoplasmic shuttling and subsequent degradation of $p27^{Kip1}$ mediated by Jab1/CSN5 and the COP9 signalosome complex. *J Biol Chem* 2002; 277:2302-10.
22. Korbonits M, Chahal HS, Kaltas G, Jordan S, Urmanova Y, Khalimova Z, Harris PE, *et al*. Expression of phosphorylated $p27^{Kip1}$ protein and Jun activation domain-binding protein 1 in human pituitary tumors. *J Clin Endocrinol Metab* 2002; 87:2635-43.
23. Sui L, Dong Y, Ohno M, Watanabe Y, Sugimoto K, Tai Y, Tokuda M. Jab1 expression is associated with inverse expression of $p27^{Kip1}$ and poor prognosis in epithelial ovarian tumors. *Clin Cancer Res* 2001; 7:4130-5.
24. Shen L, Tsuchida R, Miyauchi J, Saeki M, Honna T, Tsunematsu Y, Kato J, Mizutani S. Differentiation-associated expression and intracellular localization of cyclin-dependent kinase inhibitor $p27^{Kip1}$ and c-Jun co-activator Jab1 in neuroblastoma. *Int J Oncol* 2000; 17:749-54.
25. Sato M, Hayashi Y, Yanagawa T, Yoshida H, Yura Y, Azuma M, Ueno A. Intermediate-sized filaments and specific markers in a human salivary gland adenocarcinoma cell lines and its nude mouse tumors. *Cancer Res* 1985; 45:3878-90.
26. Yanagawa T, Hayashi Y, Yoshida H, Yura Y, Nagamine S, Bando T, Sato M. An adenoid-squamous-carcinoma-forming cell line established from an oral keratinizing squamous-cell carcinoma expressing carcinoembryonic antigen. *Am J. Pathol* 1986; 124: 496-509.
27. Hayashi Y, Yanagawa T, Yoshida H, Azuma M, Nishida T, Yura Y, Sato M. Expression of vasoactive intestinal polypeptide and amylase in a human parotid gland adenocarcinoma cell line in culture. *J Natl Cancer Inst* 1987; 79:1025-37.
28. Kawamata H, Nakashiro K, Uchida D, Harada K, Yoshida H, Sato M. Possible contribution of active MMP2 to lymp-node metastasis and secreted cathepsin L to bone invasion of newly established human oral-squamous cancer cell lines. *Int J Cancer* 1997; 70:120-7.
29. Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of tetrazolium-based semiautomated calorimetric assay: assessment of chemosensitivity testing. *Cancer Res* 1987; 47: 936-42.
30. Gupta D, Podar K, Tai YT, Lin B, Hideshima T, Akiyama M, *et al*. b-lapachone, a novel plant product, overcomes drug resistance in human multiple myeloma cells. *Exp Hematol* 2002; 7:711-20.
31. Tomoda K, Yoneda-Kato N, Fukumoto A, Yamanaka S, Kato JY. Multiple functions of Jab1 are required for early embryonic development and growth potential in mice. *J Biol Chem* 2004; 279:43013-8.
32. Oono K, Yoneda T, Manabe T, Yamagishi S, Matsuda S, Hitomi J, *et al*. Jab1 participates in unfolded protein responses by association and dissociation with IRE1. *Neurochem Int* 2004; 45:765-72.
33. Katayose Y, Kim M, Rakkar ANS, Li Z, Cowan KH, Seth P. Promoting apoptosis: a novel activity associated with the cyclin-dependent kinase inhibitor p27. *Cancer Res* 1997; 57:5441-5.
34. Shintani S, Li C, Mihara M, Hino S, Nakashiro KI, Hamakawa H. Skp2 and Jab 1 expression are associated with inverse expression of $p27^{Kip1}$ and poor prognosis in oral squamous cell carcinoma. *Oncology* 2003; 65:355-62.