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구강암세포주인 HN22에서 Dibenzylideneacetone에 의해 유도되는 세포사멸현상에서 Specificity Protein 1의 관련성에 관한 연구

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〈Abstract〉

Specificity Protein 1 is Involved in Dibenzylideneacetone-induced Apoptosis in HN22 Human Oral Cancer Cells

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Dibenzylideneacetone (DBA), an analogue of curcumin has been shown to have anti-cancer activity in a variety of tumor cell lines. However, the anti-cancer activity of DBA and its molecular mechanism in HN22 oral cancer cell line have not been fully explored. The effects of DBA on anti-proliferative and apoptotic activity were evaluated by the trypan blue exclusion assay, 4'-6-diamidino-2-phenylindole (DAPI) staining, Western blot analysis, and reverse transcriptase-polymerase chain Reaction (RT-PCR). Our data showed that the treatment of DBA to HN22 cells exerted anti-proliferative and apoptotic activities and the activity was accompanied by a decrease in Sp1 protein, Sp1 mRNA and its promoter activity. DBA also reduced the expression level of Sp1 protein and caused apoptotic cell death in HN22 cells simultaneouly. Phosphorylation of ERK and JNK were regulated by DBA whereas phosphorylation of p38 was not altered. Overall, our results suggest that the regulation of Sp1 activities and ERK/JNK are involved in DBA-induced apoptosis and DBA can be a promising anticancer drug candidate for the treatment of oral cancer.

Key words : DBA, Oral cancer cells, Sp1, ERK, JNK, Apoptosis

Ⅰ. INTRODUCTION

Specificity protein 1 (Sp1) is a zinc finger transcription factor of the Sp/Kruppel-like factor family, which regulates constitutive levels of genes involved in various physiological processes, including apoptosis, angiogenesis, and cell cycle regulation in both normal and malignant tissues[1]. Sp1 is overexpressed in many human tumors and cancer cell lines[2-5].

MAPKs consist of at least three signal transduction pathways (ERK, JNK, and p38). It is important in regulating cell proliferation and cell survival in response to growth stimulation and stress. Activation of the ERK pathway is involved in cell proliferation, whereas on the contrary, JNK and p38 kinase pathways are primarily activated by stress signals, and activation of these pathways leads to inhibition of cellular proliferation and/or decreased cell survival[6]. It

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is primarily activated by stress signals, and its activation leads to the inhibition of cellular proliferation and / or decreased cell survival[7].

Curcumin (Diferuloylmethane), the main component isolated from ancient Indian spice turmeric (plant Curcuma longa), has attracted significant research attention in recent years[8,9]. Notably, curcumin has been undergoing multiple early clinical trials owing to its novel therapeutic properties against a broad spectrum of human disorders such as cancer and inflammatory diseases[10-13]. Its analogues including dibenzylideneacetone (DBA), dibenzoylmethane (DBM), dibenzoylpropane (DBP), and (1E, 4E)-1, 5-bis (2, 3-dimethoxyphenyl) penta-1, 4-dien-3-one) also exhibited anticancer effects[14,15]. DBA inhibits the growth of melanoma in vitro and in vivo through inhibition of N-myristoyltransferase-1, abrogation of mitogen-activated protein kinase, suppression of Akt, down-regulation of STAT-3, and inhibition of S6 kinase activation[16]. However, the apoptotic activity and molecular mechanism of DBA in HN22 human oral cancer cells are not fully understood yet. In this study, we determined the apoptotic effects of DBA in HN22 cells and explored the underlying molecular signaling associated with DBA-mediated apoptosis.

II. MATERIALS AND METHODS

1. Cell Culture and Chemical Treatment

The HN22 cells were obtained from the School of Dentistry, Dankook University (Cheonan, Korea). Dulbecco's modified essential medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin, 100x antibiotic solution, Trypsin, and D-PBS was obtained from WelGENE Inc.(Dae-gu, Korea). HN22 cells were cultured in DMEM supplemented with 10% FBS and 100 U/ml each of penicillin and streptomycin in a humidified atmosphere containing 5% CO_2 at 37 ℃. An equal number of cells were seeded and allowed to attach. DBA (purity of 99%) was prepared in DMSO and stored as small aliquots at 20 ℃ and then diluted further in cell culture medium as needed.

2. Antibodies

Cleaved PARP, Cleaved caspase-3, p-ERK, ERK, p-JNK, JNK, p-p38 and p38 antibodies were obtained from Cell Signaling Technology, Inc. (Charlottesville, VA, USA). Antibodies against Sp1 and actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

3. Cell Viability Assay

The HN22 cells were treated with 5, 7.5, and 10 μM of DBA for 24 hr. The number of viable cells was counted using a hematocytometer with trypan blue (0.4%). Each experiment was carried out in triplicate and the results are expressed as the percentage of surviving cells compared to the DMSO-treated group.

4. DAPI Staining

Apoptotic cell death was determined morphologically using a fluorescent nuclear dye, DAPI. This showed the number of apoptotic cells with chromatin condensation and nuclear fragmentation. HN22 cells were incubated with DMSO or DBA (5, 7.5 and 10 μM) for 24 hr, then harvested by trypsinization, and fixed in 100% ethanol overnight at -20 ℃. The cells were re-suspended in PBS, deposited on poly-L-lysin coated slides, stained with a DAPI solution. The cell morphology was observed under a fluorescence microscope.

5. Western Blot Analysis

Whole-cell lysates were extracted with SDS lysis buffer [1M Tris-Cl (pH 7.5), 0.5M EDTA (pH8.0), 5M NaCl, 1M

DTT, 10% NP40, 0.1M PMSF, aptotinin and leupeptin] was used and quantified with DC Protein Assay kit was obtained from BIO-RAD, Inc. (Hercules. CA, USA). Equal amount of protein from each sample was mixed with 5X loading buffer and heated at 95 ℃ for 5min. Equal amount of protein was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene fluoride (PVDF) membrane was obtained from BIO-RAD, Inc. (Hercules. CA, USA). The membranes were blocked with 5% skim milk in TBST buffer at RT for 2 hr, washed with TBST and maintained overnight at 4 ℃ with primary antibody. Washing was done with TBST and incubation with horseradish peroxidase (HRP)-conjugated secondary antibody was done at RT for 2 hr. After washing with TBST, the antibody-bound proteins were detected using an ECL Western Blotting Luminol Reagent were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

6. Reverse Transcription-polymerase Chain Reaction (RT-PCR)

The mRNA expression of Sp1 was quantified by RT-PCR analysis, using β-Actin mRNA as an internal standard. Total RNA was extracted from the cells using an easy-BLUE Total RNA Extraction kit (iNtRON; Seongnam, Korea). One microgram of RNA was used to synthesize the cDNA using Reverse Transcription System (Promega, Madison, WI, USA), which was then amplified by PCR using the specific primers for Sp1; 5'-ATG GGG GCA ATG GTA ATG GTG G-3' (S), 5'-TCA GAA CTT GCT GGT TCT GTA AG-3' (AS), and β-Actin; 5'-GTG GGG CGC CCC AGG CAC CA-3'(S), 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'(AS). The PCR conditions of Sp1 was as follows: (35 cycles: 1 min at 94 ℃, 1 min at 62 ℃ and 10 min at 72 ℃), and the PCR condition of β-Actin was as follows: (28 cycles: 1 min at 94 ℃, 1 min at 60 ℃ and 10 min at 72 ℃). Equal amounts of RT-PCR products were separated on a 2% agarose gel and stained with ethidium bromide.

7. Dual-luciferase Assay

HN22 cells were plated in 12-well and transfected with 300 ng of Sp1 with LipofectAMINE 2000 reagent was obtained from Invitrogen (Carlsbad, CA, USA). Six hours after transfection the cells were treated with 5% FBS, DBA for 24 hr. HN22 cell lysates were analyzed with the Dual Luciferase Reporter Assay system was obtained from Promega (Madison, WI, USA). Relative luciferase expression was determined as the ratio of firefly to Renilla luciferase activities using a Microbeta Trilux 1450 luminescence counter was purchased from Perkin Elmer (Turku, Finland). Transfections were performed in triplicate, and the mean and standard error were calculated for each condition.

8. Statistical Analysis

The data were assessed for statistical significance using a Student's t-test. A value of $p < 0.05$ compared to the vehicle control was considered statistically significant.

Ⅲ. RESULTS

1. DBA Decreases Cell Viability and Induces Apoptosis in HN22 Cells

To investigate the growth-inhibitory effect of DBA in HN22 cells, the observation of morphological change by optic microscope and a trypan blue exclusion assay were performed. The results showed that cells were detached and cell viability was decreased by DBA in a concentration-dependent manner (Fig. 1A). Induction of apoptosis by DBA was further evaluated by DAPI staining and Western blot analysis. DBA clearly induced the cleavage of PARP and caspase-3 in a concentration-dependent manner (Fig.

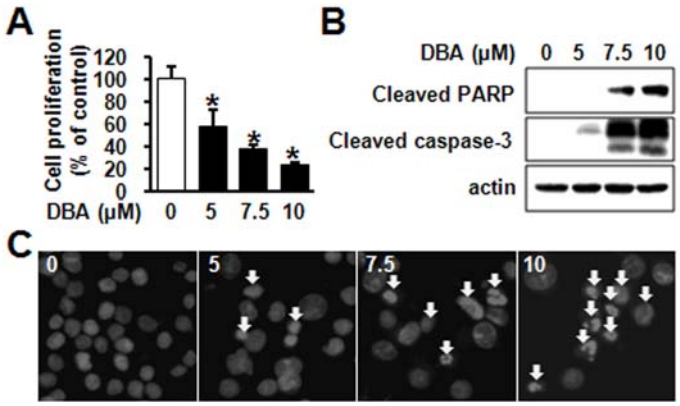

Fig. 1. The effect of DBA on cell viability and apoptosis in HN22 cells

HN22 cells were treated with 5, 7.5, or 10 μM of DBA for 24 hr. Cell viability was determined using a trypan blue exclusion assay (A). The apoptotic effect of DBA was determined by Western blot analysis using the antibodies against cleaved PARP and cleaved caspase-3 (B) and DAPI staining (C). Results are expressed as means ± SD for triplicate experiments and significance ($P < 0.05$) compared with DMSO-treated cells is given (*).

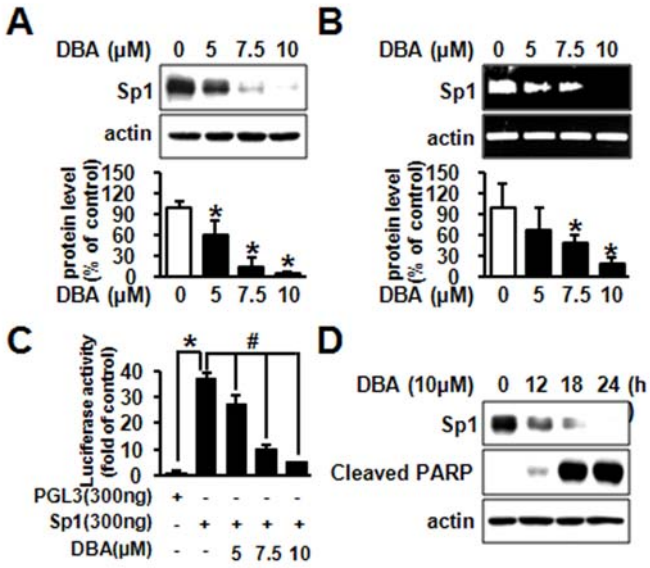

Fig. 2. The effects of DBA on Sp1 in HN22 cells

The effect of DBA on Sp1 protein levels were determined by Western blot analysis in HN22 cells for 24 hr (A). Sp1 mRNA levels were confirmed by RT-PCR in HN22 cells and normalized to β-actin (B). HN22 cells were transfected with PGL3 vector control (white bar) or Sp1 promoter construct (black bar), treated with DBA, and induction of luciferase activity was determined (C). Sp1 protein expression was determined by Western blot analysis in HN22 cells were treated with 10 μM of DBA for 12, 18 or 24 hr (D). All data are expressed as means ± SD for triplicate experiments and significance ($P < 0.05$) compared with DMSO-treated cells is indicated (*).

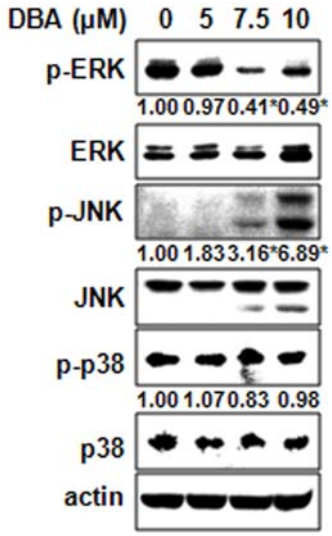

Fig. 3. The effect of DBA on MAPKs pathway in HN22 cells

HN22 cells were treated with DMSO or various concentrations of DBA for 24 hr and p-ERK, ERK, p-JNK, JNK, p-p38 and p38 expression were analyzed by Western blot analysis. All data are expressed as means ± SD for triplicate experiments and significance ($P < 0.05$) compared with DMSO-treated cells is indicated (*).

1B). As shown in Fig. 1C, DBA increased condensed and fragmented nuclei compared to DMSO (vehicle control). Overall, these findings suggested that DBA can induce apoptosis to alleviate the viability of HN22 cells.

2. DBA decreases Sp1 protein at the transcriptional level in HN22 cells

Because Sp1 protein plays an important role in the growth of cancer[1], experiments were carried out to determine whether DBA treatment affects its expression level. The results in Fig. 2A and 2B showed the down-regulation of Sp1 protein and mRNA by DBA in a concentration-dependent manner. DBA also inhibited the transactivation of Sp1 promoter in HN22 cells (Fig. 2C). The time-course effects of DBA on Sp1 protein and PARP cleavage were investigated. The results showed that the time-course inhibition of Sp1 protein and cleavage of PARP by DBA were observed after treatment for 12 hr, and this response remains down-regulated for up to 24 hr (Fig. 2D). Thus, the results suggest that DBA may induce apoptosis through the down-regulation of Sp1 protein at the transcriptional level in HN22 cells.

3. DBA decreased phosphorylation of ERK1/2 and increased phosphorylation of JNK but did not cause any changes in p38 levels

Because DBA was previously identified that it can regulate MAPK signaling[17]. Therefore, we analyzed expression of phospho-ERK1/2, JNK, and p38 in HN22 cells. DBA decreased the phosphorylation of ERK1/2 and increased the phosphorylation of JNK but phospho-p38 was not altered (Fig. 3). There was no change in the total form of MAPKs, as measured by Western blotting. These data indicate that ERK1/2 and JNK may be associated with DBA-regulated apoptosis in HN22 cells.

Ⅳ. DISCUSSION

These results indicate that dibenzylideneacetone (DBA) markedly induces cell death in HN22 human oral cancer cell line. Our results also provide detailed molecular mechanistic information as to how DBA exerts its apoptotic effects on HN22 human oral cancer cells.

DBA is one such agent that has been shown to inhibit cell growth and induce apoptosis in several cancer cell types[17-20]. However, the apoptotic activity and molecular mechanism of DBA were not fully understood yet in HN22 cells. In this study, we investigated the apoptotic activity of DBA in HN22 cells. Our results showed that DBA resulted in decreased cell viability and induced apoptotic cell death. Consistent with other previous studies[19,20], DBA may thus be a potent apoptotic inducer in HN22 cells.

Sp1 is a member of the mammalian transcription factor family that binds to GC-rich sites containing GC-boxes. Recently, it was reported that Sp1 protein is over-expressed in many human tumors and cancer cell lines[2,3,21-23]. Our previous studies found that Sp1 was highly expressed in human oral tumor tissues and cell lines, and the down-regulation of Sp1 is deeply related to growth inhibition and induction of apoptosis in oral, prostate, and cervical cancer cell lines[24-27]. Sp1 has also been implicated in multiple cell processes, including apoptosis, through activation of the FAS-ligand[28,29]. Our laboratory also found that DBA-induced apoptosis was associated with down-regulation of Sp1 in human oral cancer cell lines[19,20]. Therefore, we examined the effects of DBA on the expression of Sp1 as the key molecular factors in human oral cancer cells. Our results show that DBA decreases the expression levels of Sp1 proteins, suggesting that DBA inhibits the growth and induces apoptosis in HN22 cells through the regulation of Sp1.

MAPKs are serine/threonine superfamily kinases consisting of ERK1/2, JNK, and p38 MAPK that are involved

in a range of cellular programs including cell growth, cell differentiation, cell development, inflammation, and apoptosis[30]. Modulation of the ERK pathway is associated with certain cellular responses, such as regulation of the cell cycle, apoptosis, proliferation, intercellular communication via gap junction, and signaling molecules depending on cell type, strength, and duration of the signal[31-33]. The critical role of JNK has been shown in the lethal effects of diverse cytotoxic stimuli, including ceramide, Fas ligand, UV, among others[34-36]. Previous studies also found that Sp1 levels are regulated by affecting MAPK signaling in PC-3, PC-3N and DU-145 cells[37]. Thus, we investigated the effects of DBA on MAPKs proteins (ERK1/2, JNK and p38) in HN22 cells and the results showed that DBA affected ERK and JNK, but did not affect phospho-p38 suggesting Induction of apoptosis by DBA may be associated with the modification of ERK and JNK pathways.

In summary, DBA decreases cell viability and induces apoptosis of HN22 cells by inhibiting Sp1 at the transcription level and it regulates phosphorylation of ERK1/2 and JNK in HN22 cells. Therefore, we suggest that DBA may be a promising anticancer drug candidate for the treatment of oral cancer. However, further direction using animal model is necessary before clinical application of DBA.

Ⅵ. REFERENCES

1. Black AR, Black JD, Azizkhan-Clifford J: Sp1 and kruppel-like factor family of transcription factors in cell growth regulation and cancer. J Cell Physiol 2001;188: 143-160.
2. Zannetti A, Del Vecchio S, Carriero MV, et al: Coordinate up-regulation of Sp1 DNA-binding activity and urokinase receptor expression in breast carcinoma. Cancer Res 2000;60:1546-1551.
3. Wang L, Wei D, Huang S, et al: Transcription factor Sp1 expression is a significant predictor of survival in human gastric cancer. Clin Cancer Res 2003;9:6371-6380.
4. Chadalapaka G, Jutooru I, Chintharlapalli S, et al: Curcumin decreases specificity protein expression in bladder cancer cells. Cancer Res 2008;68:5345-5354.
5. Chintharlapalli S, Papineni S, Ramaiah SK, et al: Betulinic acid inhibits prostate cancer growth through inhibition of specificity protein transcription factors. Cancer Res 2007;67:2816-2823.
6. Yu R, Jiao JJ, Duh JL, et al: Phenethyl isothiocyanate, a natural chemopreventive agent, activates c-Jun N-terminal kinase 1. Cancer Res 1996;56:2954-2959.
7. Chang L, Karin M: Mammalian MAP kinase signalling cascades. Nature 2001;410:37-40.
8. Stix G: Spice Healer. Sci Am 2007;296:54-57.
9. Sharma RA, Gescher AJ, Steward WP: Curcumin: the story so far. Eur J Cancer 2005;41:1955-1968.
10. Aggarwal BB, Kumar A, Bharti AC: Anticancer potential of curcumin: preclinical and clinical studies. Anticancer Res 2003;23:363-398.
11. Egan ME, Pearson M, Weiner SA, et al: Curcumin, a major constituent of turmeric, corrects cystic fibrosis defects. Science 2004;304:600-602.
12. Chen H, Zhang ZS, Zhang YL, et al: Curcumin inhibits cell proliferation by interfering with the cell cycle and inducing apoptosis in colon carcinoma cells. Anticancer Res 1999;19:3675-3680.
13. Bharti AC, Donato N, Singh S, et al: Curcumin (diferuloylmethane) down-regulates the constitutive activation of nuclear factor-kappa B and IkappaBalpha kinase in human multiple myeloma cells, leading to suppression of proliferation and induction of apoptosis. Blood 2003;101:1053-1062.
14. Anand P, Sung B, Kunnumakkara AB, et al: Suppression of pro-inflammatory and proliferative pathways by diferuloylmethane (curcumin) and its analogues dibenzoylmethane, dibenzoylpropane, and

dibenzylideneacetone: role of Michael acceptors and Michael donors. Biochem Pharmacol 2011;82:1901-1909.

15. Wang Y, Xiao J, Zhou H, et al: A novel monocarbonyl analogue of curcumin, (1E, 4E)-1, 5-bis(2,3- dimethoxyphenyl) penta-1, 4-dien-3-one, induced cancer cell H460 apoptosis via activation of endoplasmic reticulum stress signaling pathway. J Med Chem 2011;54:3768-3778.
16. Bhandarkar SS, Bromberg J, Carrillo C, et al: Tris (dibenzylideneacetone) dipalladium, a N-myristoyltransferase-1 inhibitor, is effective against melanoma growth in vitro and in vivo. Clin Cancer Res 2008;14:5743-5748.
17. Mullally JE, Fitzpatrick FA: Pharmacophore model for novel inhibitors of ubiquitin isopeptidases that induce p53-independent cell death. Mol Pharmacol 2002;62: 351-358.
18. Prasad S, Yadav VR, Ravindran J, et al: ROS and CHOP are critical for dibenzylideneacetone to sensitize tumor cells to TRAIL through induction of death receptors and downregulation of cell survival proteins. Cancer Res 2011;71:538-549.
19. Yu HJ, Shin JA, Nam JS, et al: Apoptotic effect of dibenzylideneacetone on oral cancer cells via modulation of specificity protein 1 and Bax. Oral Dis 2013;19:767-774.
20. Lee HE, Choi ES, Jung JY, et al: Inhibition of specificity protein 1 by dibenzylideneacetone, a curcumin analogue, induces apoptosis in mucoepidermoid carcinomas and tumor xenografts through Bim and truncated Bid. Oral Oncol 2014;50:189-195.
21. Chiefari E, Brunetti A, Arturi F, et al: Increased expression of AP2 and Sp1 transcription factors in human thyroid tumors: a role in NIS expression regulation? BMC Cancer 2002;2:35.
22. Hosoi Y, Watanabe T, Nakagawa K, et al: Up-regulation of DNA-dependent protein kinase activity and Sp1 in colorectal cancer. Int J Oncol 2004;25:461-468.
23. Yao JC, Wang L, Wei D, et al: Association between expression of transcription factor Sp1 and increased vascular endothelial growth factor expression, advanced stage, and poor survival in patients with resected gastric cancer. Clin Cancer Res 2004;10:4109-4117.
24. Choi ES, Shim JH, Jung JY, et al: Apoptotic effect of tolfenamic acid in androgen receptor-independent prostate cancer cell and xenograft tumor through specificity protein 1. Cancer Sci 2011;102:742-748.
25. Shim JH, Shin JA, Jung JY, et al: Chemopreventive effect of tolfenamic acid on KB human cervical cancer cells and tumor xenograft by downregulating specificity protein 1. Eur J Cancer Prev 2011;20:102-111.
26. Shin JA, Shim JH, Jeon JG, et al: Apoptotic effect of Polygonum Cuspidatum in oral cancer cells through the regulation of specificity protein 1. Oral Dis 2011;17:162-170.
27. Shin JA, Kim JJ, Choi ES, et al: In vitro apoptotic effects of methanol extracts of Dianthus chinensis and Acalypha australis L. targeting specificity protein 1 in human oral cancer cells. Head Neck 2013;35:992-998.
28. Kavurma MM, Santiago FS, Bonfoco E, et al: Sp1 phosphorylation regulates apoptosis via extracellular FasL-Fas engagement. J Biol Chem 2001;276:4964-4971.
29. Sun Y, Giacalone NJ, Lu B: Terameprocol (tetra-O-methyl nordihydroguaiaretic acid), an inhibitor of Sp1-mediated survivin transcription, induces radiosensitization in non-small cell lung carcinoma. J Thorac Oncol 2011;6:8-14.
30. Chan-Hui PY, Weaver R: Human mitogen-activated protein kinase kinase kinase mediates the stress-induced activation of mitogen-activated protein kinase cascades. Biochem J 1998;336:599-609.
31. Chang F, Steelman LS, Shelton JG, et al: Regulation of cell cycle progression and apoptosis by the Ras/Raf/MEK/ERK pathway (Review). Int J Oncol 2003;22:469-480.
32. Agell N, Bachs O, Rocamora N, et al: Modulation of the Ras/Raf/MEK/ERK pathway by Ca(2+), and

calmodulin. Cell Signal 2002;14:649-654.

33. Trosko JE, Chang CC: Mechanism of up-regulated gap junctional intercellular communication during chemoprevention and chemotherapy of cancer. Mutat Res 2001;480-481:219-229.
34. Verheij M, Bose R, Lin XH, et al: Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. Nature 1996;380:75-79.
35. Wilson DJ, Fortner KA, Lynch DH, et al: JNK, but not MAPK, activation is associated with Fas-mediated apoptosis in human T cells. Eur J Immunol 1996;26: 989-994.
36. Zanke BW, Boudreau K, Rubie E, et al: The stress-activated protein kinase pathway mediates cell death following injury induced by cis-platinum, UV irradiation or heat. Curr Biol 1996;6:606-613.
37. Sroka IC, Nagle RB, Bowden GT: Membrane-type 1 matrix metalloproteinase is regulated by sp1 through the differential activation of AKT, JNK, and ERK pathways in human prostate tumor cells. Neoplasia 2007;9:406-417.