

Anticancer Effects of Astaxanthin and α -tocopherol in Esophageal Cancer Cell Lines

Background/Aims: Astaxanthin (AX) has been attributed with potential for protecting the organism against different types of cancer due to its anti-oxidant activity. Also several *in vivo* and *in vitro* studies suggest certain naturally occurring vitamin E (i.e. α -tocopherol) as promising anticancer agents. We assessed the effect of AX and α -tocopherol (AT) respectively and their combination on human esophageal cancer cell lines to investigate the mechanism of anticancer effect and their therapeutic potential. **Materials and Methods:** Two human esophageal cancer cell lines (TE-1, TE-4) were exposed to AX (6 to 10 μ g/mL) and AT (20 to 100 μ M) for 24 hours. Quantification of proliferation was performed by MTT assay. Cell cycle machinery proteins such as p-AKT, p-p38, cyclin D1, p27 and caspase-3 were investigated by Western blot. **Results:** Significant inhibition of cell proliferation of AX and AT was observed in TE-4 cell line by a dose-dependent manner. Furthermore, AX and AT as single agents increased the protein expression of p27 and cleaved caspase-3 in TE-4 cell line. The combination of the two agents decreased the expression of cyclin D1, however they did not demonstrate pro-apoptotic effect. **Conclusions:** AX and AT as single agents are effective at inhibition of cell proliferation and induce apoptosis by the modulation of cell cycle machinery proteins in esophageal cancer cell lines. However, our data could not suggest that their combination has any cooperative apoptotic effect. (Korean J Helicobacter Up Gastrointest Res 2011;11:170-175)

Key Words: Astaxanthin; Alpha-tocopherol; Esophageal cancer; Cell cycle proteins

Department of Gastroenterology, Korea University, Guro and Ansan Hospital, Seoul, Korea

Sang-ah Lim, Joon Young Lee, Won Ho Jung, Eun Hye Lim, Moon Kyung Joo, Beom Jae Lee, Jong-Jae Park, Jae Seon Kim, Young-Tae Bak, Sung Woo Jung, Sang Woo Lee

Received : June 17, 2011

Accepted : November 23, 2011

Corresponding author : Jong-Jae Park
Department of Internal Medicine, Guro Hospital, Korea University College of Medicine, 80, Guro-dong 2-ga, Guro-gu, Seoul 152-703, Korea
Tel: 02-2626-3003
Fax: 02-854-8453
E-mail: gi7pjj@yahoo.co.kr

INTRODUCTION

There is abundant laboratory evidence that carotenoids possess potent cancer chemopreventive properties^{1,2} independent of their antioxidant activity or potential for conversion to retinoids.^{3,4} Recently, the red ketocarotenoid astaxanthin (AX), 3, 3'-dihydroxy- β , β -carotene-4,4'-dione, which is main carotenoid in aquatic animals and in seafoods has been found. It does not possess a pro-vitamin A activity but attract considerable interest because of its potent antitumoral, antioxidant and immunomodulatory activities. They are distinctly different and, at least in some cases, more potent than that of β -carotene and other carotenoids.⁵⁻⁹

Increasing evidence suggests that AX is a potent antitumoral

agent in various human cancer cell lines. Dietary AX exerted antitumoral activity in the post-initiation phase of carcinogen-induced oral cancer models.⁷ Rats fed a carcinogen (4-Nitroquinoline-1-oxidase) but supplemented with astaxanthin had a significantly lower incidence of different types of cancerous growth in their mouths than rats fed only the carcinogen by decreasing cell proliferation of nonlesional squamous epithelium, and such effects may be partly due to suppression of cell proliferation.⁷ On the other hand, the effects of AX have been studied in human breast cancer cells,¹⁰ in which AX inhibits the proliferation of MCF-7 cell line and downregulates bcl-2 gene expression. Also, AX inhibited cell growth in a dose- and time-dependent manner, by arresting cell cycle progression and by promoting apoptosis in HCT-116 colon cancer cells.⁴ And

reduced gastric inflammation via modulation of inflammatory cytokines in a *Helicobacter pylori* infected mice model.¹¹

Meanwhile, α -tocopherol (AT) is a promising anti-cancer micronutrient that has been shown to promote tumor dormancy, apoptosis and inhibit cellular proliferation in breast cancer, melanoma and colon cancer.¹²⁻¹⁴ Furthermore, AT showed inhibition of cell proliferation, induction of cell cycle arrest and apoptosis as a single agent or 2- to 3-drug combination.¹⁵ It is interesting that dietary AT exerted antitumoral activity in the post-initiation phase of carcinogen-induced oral cancer models.

We previously performed an *in vitro* study using synthetic AX which showed cell cycle arrest and growth inhibition in gastric cancer cell lines.¹⁶ Moreover, recent *in vivo* study reported that dietary AX in combination with AT had cooperative inhibitory effect on oxidative stress in diabetic rat model.¹⁷ With this background, we designed this study to examine the effects of AX and AT respectively and double combination on the growth of different human esophageal cancer cells and to investigate some molecular pathways involved in cell cycle progression, apoptosis and cell survival.

MATERIALS AND METHODS

1. Materials

Two human esophageal cancer cell lines (TE-1 and TE-4) were used in this study. Both cell lines were established from the primary esophageal squamous cell carcinomas and obtained from the Riken Biosource Center (Tsukuba, Ibaraki, Japan). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum containing 1% penicillin-streptomycin in a 37°C, 5% CO₂ humidifier incubator.

2. Reagents

AX obtained from natural extracts was provided by Dr. Seok-Keun Choi and Sun-Joong Kim (College of Life Science & Biotechnology, Korea University) and commercial AT (Sigma Chemical Inc., St. Louis, MO, USA) was purchased. AX was dissolved in 100 μ L methanol, and AT was dissolved in dimethyl sulfoxide (DMSO) as stock solutions and then diluted in DMEM/F12 medium immediately before use.

3. MTT assay

Both cells were inoculated in 96-well plate at a density of 5×10^3 cells/well overnight prior to drug treatment. Subsequent-

ly, drugs were added as single agents at various concentrations (0.5 ~ 50 μ g/mL for AX, 5 ~ 150 μ M for AT), followed by incubation at 37°C, 5% CO₂ for 24 hours. After this, 20 μ L of MTT solutions dissolved in 5 mg/mL PBS were added in each wells and additionally were incubated for 2 hours at 37°C in culture hood. Then we removed media and added 20 μ L of DMSO in each well. The resultant product was quantified by spectrophotometry using a plate reader at 570 nm using reference at 670 nm. All experiments were repeated 3 times.

4. Western blot analysis

Each two esophageal cancer cells were incubated in 100 mm dish until 80% growth followed by the treatment. After drugs were added as single agents and as combination at 10 μ g/mL for AX, 100 μ M for AT, they were incubated at 37°C, 5% CO₂ for 24 hours. Drug treated TE-4 cells were harvested, washed once with icecold phosphate buffered saline (PBS) and gently lysed for 30 min in ice-cold lysis buffer (1 mM MgCl₂, 350 mM NaCl, 20 mM HEPES, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1mM Na₄P₂O₇, 1 mM PMSF, 1 mM aprotinin, 1.5 mM leupeptin, 1 mM Na₃VO₄, 20% glycerol, 1% NP40). Cell lysates were centrifuged for 10 min at 4°C and protein was separated on 8% to 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel, transferred onto a polyvinylidene fluoride membrane (Millipore Co., Bedford, MA, USA), and probed with primary antibodies. The anti-p-AKT, anti-p-p38, anti-cyclin D1 monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-total-caspase-3 and anti-cleaved-caspase-3 monoclonal antibodies were purchased from Imgenex (San Diego, CA, USA). Anti- β -actin antibody (Sigma Chemical Inc., St. Louis, MO, USA) was used as sample loading control. The blots were washed with PBS and exposed to horseradish peroxidase-labelled secondary antibodies for 45 min at room temperature. The immunocomplexes were visualized by the enhanced chemiluminescence detection system (ECLTM Western blotting Analysis System, Amersham, Buckinghamshire, UK) and quantified by densitometric scanning.

RESULTS

1. Effects of AX and AT treatment on cell growth in human esophageal cancer cells

Using MTT assay, cell death was monitored with physio-

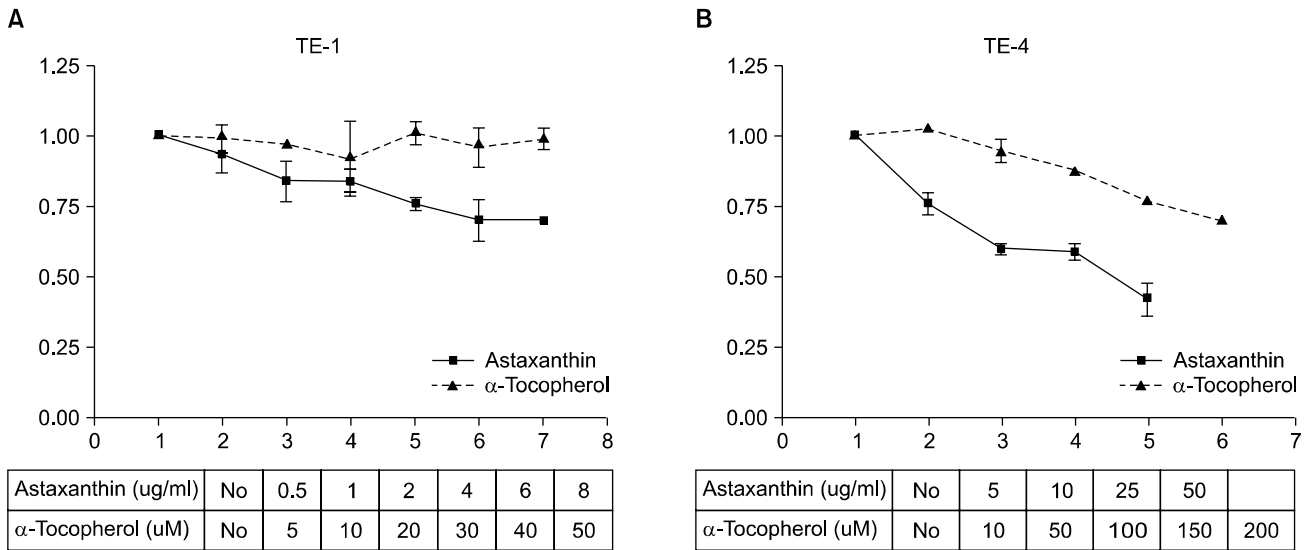


Fig. 1. Effects of Astaxanthin (AX) and α -tocopherol (AT) on the growth of human esophageal cancer cell lines (TE-1, TE-4) using MTT assay. (A, B) shows the growth-inhibitory effects of AX (0.5~50 μ g/mL) and AT (5~150 μ M) in TE-1 and TE-4 cell lines treated for 24 hours.

logical and pharmacological concentrations of AX and AT after 24 hours of treatment. The effects of the AX and AT on the growth of human esophageal cancer cells are shown in Fig. 1. In treating different doses of the astaxanthin (0.5~50 μ g/mL) and α -tocopherol (5~150 μ M) in TE-1 and TE-4 cell lines treated for 24 hours, MTT assay showed that AX and AT as single agents demonstrated a dose-dependent inhibition of cell proliferation in TE-4 cell line. For this cell line, concentrations of AX and AT higher than 10 μ g/mL and 100 μ M were considered to be effective in inducing cell growth inhibition. On the other hand, inhibitory effect of AX and AT for cell growth was minimal in TE-1 cell line.

2. Effects of AX and AT treatment on cell progression and apoptosis in human esophageal cancer cells

To understand the mechanisms of cell growth inhibition by AX and AT, we further investigated the effects of AX, AT and their combination on the cell cycle and apoptosis related proteins. TE-4 cell line was treated with AX and AT as either single agents or their combination for 24 hours. The fixed concentrations for AX (10 μ g/mL) and AT (100 μ M) were chosen for treatment of cells. Immunoblotting was performed to detect the activation of p-AKT, p-p38, cyclin D1, p27 and caspase-3. First, AX as a single agent decreased the expression of p-AKT. However, AT alone or combination of AX and AT increased p-AKT. AX alone also increased the expression of p-p38.

Cyclin D1 was activated by AX or AT as a single agent, however decreased when both were combined. Importantly, p27 and cleaved-caspase-3 were decreased by AX or AT alone, however, their combination could not exhibit such effect (Fig. 2).

DISCUSSION

Our finding shows that AX and AT may act as growth-inhibitory effect *in vitro* by dose-dependent manner in TE-4 cell line. Several previous *in vitro* studies reported that purified AX exhibited growth inhibitory effects in various cancer cell lines, including colon,¹⁸ oral,⁷ fibrosarcoma,¹⁹ breast^{10,20,21} or prostate cancer.²² Also, AT as a single agent or combination with other chemopreventive agents inhibited cellular proliferation and induced apoptosis in cell lines of gastrointestinal tract cancer.^{15,23} Our results confirmed the growth inhibitory effect of AX and AT in TE-4 esophageal cancer cell line. However, the fixed concentration of AT (100 μ M) which inhibited cellular proliferation was higher than that of other study (15 μ M) which showed such effect in squamous head and neck cancer cell line.¹⁵ Our findings also suggested that AX and AT as single agents induce apoptosis by the modulation of cell cycle machinery protein in esophageal cancer cell line. It is well known that overexpression of cyclin D1 is most frequently associated with human malignancy and down-regulation of cyclin D1 and concomitant increase in p27 involves the arrest of cell cycle

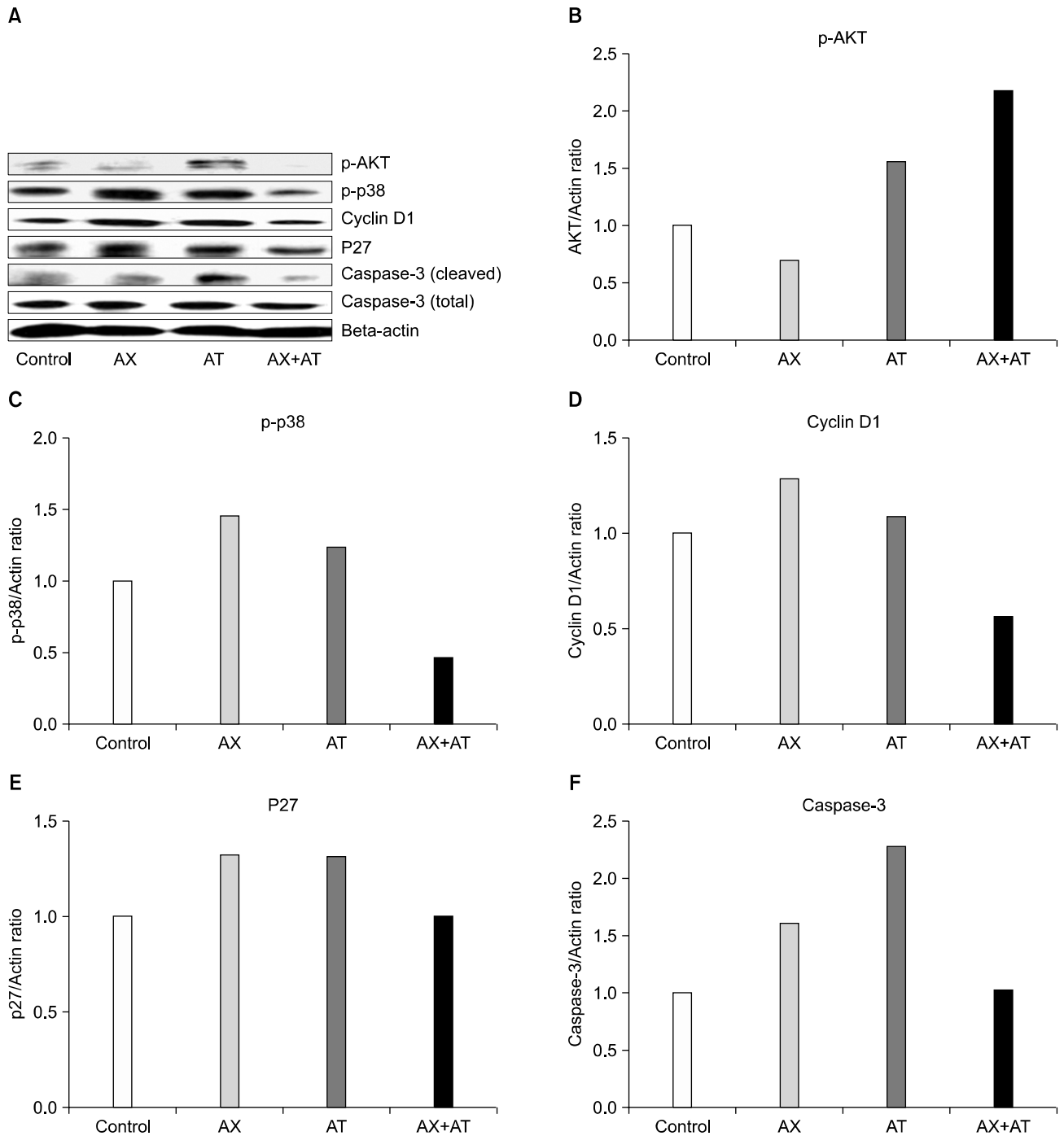


Fig. 2. p-AKT, p-p38, Cyclin-D1, p27^{kip} and Caspase-3 expression in TE-4 cell line treated with Astaxanthin (AX) and α -tocopherol (AT) for 24 hours. (A) shows representative Western Blot analyses and panels (B~F) shows densitometric analysis of five different determinations. Values are expressed as protein/actin ratio.

progression at the G0/G1 phase.²⁴ Our study showed that AX and AT as a single agents increased the expression of p27, however could not demonstrate accompanying decreased activity of cyclin D1. In contrast, their combination decreased the expression of cyclin D1, however could not accompany de-

creased activity of p27. To make a mechanism involving as arrest of cell cycle by AX or AT, further western blot analysis of p21^{waf/cip} or p53, and cell cycle analysis using FACS flow cytometer need to be performed.

Recent evidence suggests that AKT signaling pathway

changes in cell ability to undergo apoptosis and carotenoids may modulate AKT pathway in cancer cells.²⁵ In our study, AX alone and induced a decrease in phosphorylated AKT at concentrations of 10 μ g/mL, which is consistent with the pro-apoptotic effect. Additionally, AX and AT in turn increase the activity of caspase-3. However, there was discrepancy in alteration of p-AKT and caspase-3 by the treatment of AT and AX/AT combination. Further analysis of apoptosis induction is needed by investigating the alterations of inhibitors of programmed cell death (Bcl-2, Bcl-XL), pro-apoptotic agent (Bax), other caspase series (caspase-8, 9, 10) and its target protein (poly-ADP-ribose polymerase (PARP)).

There are three kinases, major MAPKs, JNK, p38 and ERK, which have been shown to regulate apoptosis.²⁶ Moreover, MAPKs signaling cascades has been reported that it play an important role in oxidative stress-induced apoptotic cell death.²⁷ In our study, AX increased the expression of phosphorylated p-38, suggesting a role of pro-oxidant and pro-apoptotic effect. Previous *in vitro* study also showed that carotenoids may exert a prooxidant in cancer cells,² and our data correspond with existing theory.

In summary, AX and AT as a single agent showed a dose-dependent inhibition of cell proliferation in TE-4 cell line. Furthermore, they as a single agent also decreased the expression of p27 and cleaved-caspase-3. The expression of cyclin D1 was decreased by the combination of both agents, however their combination could not exhibit more effect of cell cycle arrest or apoptosis. Further studies are needed to understand whether or not combination of AX and AT may be of any benefit in helping apoptosis of esophageal cancer cells.

REFERENCES

1. Palozza P, Serini S, Maggiano N, et al. Beta-Carotene down-regulates the steady-state and heregulin-alpha-induced COX-2 pathways in colon cancer cells. *J Nutr* 2005;135:129-136.
2. Palozza P, Serini S, Di Nicuolo F, Calviello G. Modulation of apoptotic signalling by carotenoids in cancer cells. *Arch Biochem Biophys* 2004;430:104-109.
3. Khachik F, Beecher GR, Smith JC Jr. Lutein, lycopene, and their oxidative metabolites in chemoprevention of cancer. *J Cell Biochem Suppl* 1995;22:236-246.
4. Palozza P, Torelli C, Boninsegna A, et al. Growth-inhibitory effects of the astaxanthin-rich *alga Haematococcus pluvialis* in human colon cancer cells. *Cancer Lett* 2009;283:108-117.
5. Terao J. Antioxidant activity of beta-carotene-related carotenoids in solution. *Lipids* 1989;24:659-661.
6. Naguib YM. Antioxidant activities of astaxanthin and related carotenoids. *J Agric Food Chem* 2000;48:1150-1154.
7. Tanaka T, Makita H, Ohnishi M, Mori H, Satoh K, Hara A. Chemoprevention of rat oral carcinogenesis by naturally occurring xanthophylls, astaxanthin and canthaxanthin. *Cancer Res* 1995;55:4059-4064.
8. Palozza P, Krinsky NI. Astaxanthin and canthaxanthin are potent antioxidants in a membrane model. *Arch Biochem Biophys* 1992;297:291-295.
9. Okuzumi J, Nishino H, Murakoshi M, et al. Inhibitory effects of fucoxanthin, a natural carotenoid, on N-myc expression and cell cycle progression in human malignant tumor cells. *Cancer Lett* 1990;55:75-81.
10. Li Z, Wang Y, Mo B. The effects of carotenoids on the proliferation of human breast cancer cell and gene expression of bcl-2. *Zhonghua Yu Fang Yi Xue Za Zhi* 2002;36:254-257.
11. Bennedsen M, Wang X, Willén R, Wadström T, Andersen LP. Treatment of *H. pylori* infected mice with antioxidant astaxanthin reduces gastric inflammation, bacterial load and modulates cytokine release by splenocytes. *Immunol Lett* 1999;70:185-189.
12. Malafa MP, Neitzel LT. Vitamin E succinate promotes breast cancer tumor dormancy. *J Surg Res* 2000;93:163-170.
13. Malafa MP, Fokum FD, Mowlavi A, Abusief M, King M. Vitamin E inhibits melanoma growth in mice. *Surgery* 2002;131:85-91.
14. Barnett KT, Fokum FD, Malafa MP. Vitamin E succinate inhibits colon cancer liver metastases. *J Surg Res* 2002;106:292-298.
15. Zhang X, Chen ZG, Khuri FR, Shin DM. Induction of cell cycle arrest and apoptosis by a combined treatment with 13-cis-retinoic acid, interferon-alpha2a, and alpha-tocopherol in squamous cell carcinoma of the head and neck. *Head Neck* 2007;29:351-361.
16. Lee BJ, Park JJ, Jung SJ, et al. S2043 Anticancer effects of astaxanthin in stomach cancer cell lines. *Gastroenterology* 2009;136:A-319.
17. Nakano M, Onodera A, Saito E, et al. Effect of astaxanthin in combination with alpha-tocopherol or ascorbic acid against oxidative damage in diabetic ODS rats. *J Nutr Sci Vitaminol (Tokyo)* 2008;54:329-334.
18. Tanaka T, Kawamori T, Ohnishi M, et al. Suppression of azoxymethane-induced rat colon carcinogenesis by dietary administration of naturally occurring xanthophylls astaxanthin and canthaxanthin during the postinitiation phase. *Carcinogenesis* 1995;16:2957-2963.
19. Jyonouchi H, Sun S, Iijima K, Gross MD. Antitumor activity of astaxanthin and its mode of action. *Nutr Cancer* 2000;36:59-65.
20. Chew BP, Park JS, Wong MW, Wong TS. A comparison of the anticancer activities of dietary beta-carotene, canthaxanthin and astaxanthin in mice *in vivo*. *Anticancer Res* 1999;19:1849-1853.
21. Ben-Dor A, Steiner M, Gheber L, et al. Carotenoids activate the antioxidant response element transcription system. *Mol Cancer Ther* 2005;4:177-186.
22. Anderson ML. Preliminary investigation of the enzymatic inhibition of 5alpha-reduction and growth of prostatic carcinoma cell line LNCap-FGC by natural astaxanthin and Saw Palmetto lipid extract *in vitro*. *J Herb Pharmacother* 2005;5:17-26.
23. Campbell SE, Stone WL, Lee S, et al. Comparative effects of

- RRR-alpha- and RRR-gamma-tocopherol on proliferation and apoptosis in human colon cancer cell lines. *BMC Cancer* 2006; 6:13.
24. Diehl JA. Cycling to cancer with cyclin D1. *Cancer Biol Ther* 2002;1:226-231.
25. Palozza P, Sheriff A, Serini S, et al. Lycopene induces apoptosis in immortalized fibroblasts exposed to tobacco smoke condensate through arresting cell cycle and down-regulating cyclin D1, pAKT and pBad. *Apoptosis* 2005;10:1445-1456.
26. Schaeffer HJ, Weber MJ. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol Cell Biol* 1999; 19:2435-2444.
27. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact* 2006;160:1-40.