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Carboxylic Derivatives of Vitamin K2 Inhibit Hepatocellular Carcinoma Cell Growth through Caspase/Transglutaminase-Related Signaling Pathways

Xian-Yang QIN[1], Shinya FUJII[2,3], Akitaka SHIMIZU[2], Hiroyuki KAGECHIKA[2] and Soichi KOJIMA[1,*]

[1] *Micro-Signaling Regulation Technology Unit, RIKEN Center for Life Science Technologies, Wako, Saitama 351–0198, Japan*
[2] *Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Tokyo 101–0062, Japan*
[3] *Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo 113–0032, Japan*

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Summary Chemoprevention of hepatocellular carcinoma (HCC) is one of the most challenging aspects of medical research. Vitamin K2 (VK2) has been suggested for its chemopreventive role in treatment of HCC, while inconsistent results in clinical trials have been reported. The present study was initiated to add to our insight into the anti-HCC cell proliferative effect of VK2 and its derivatives from a viewpoint of chemical structure. No significant effect was observed with original VK2, while VK2 derivatives bearing both isoprene units and a carboxyl-terminated side chain dose-dependently inhibited the growth of HCC cells without affecting normal liver cells. Loss-of-function analyses revealed that the anti-HCC cell activity by the VK2 derivatives was not mediated by a VK2 binding protein Bcl-2 homologous antagonist/killer (Bak) but rather associated with caspase/transglutaminase-related signaling pathways. Further studies on the carboxylic derivatives of VK2 bearing isoprene structural units introduced in this study might shed new light on the systemic treatment and prevention of HCC.

Key Words vitamin K2, hepatocellular carcinoma, chemoprevention, carboxylic group, isoprene units

Hepatocellular carcinoma (HCC), the most common type of liver cancer, is a highly lethal tumor with nearly 600,000 deaths each year worldwide (*1*). HCC recorded the worst prognosis among all cancers in both men and women in China. Only around 10% of patients survived 5 y after diagnosis due to limited treatment options and a high recurrence rate (*2*). Therefore, there is an increasing interest in the strategies for cancer chemoprevention, which is defined as the use of natural, synthetic or biological agents to prevent the incidence and improve the prognosis of intractable cancers such as HCC (*3*). One approach with enormous potential is nutritional chemoprevention emphasizing the use of dietary agents such as vitamins and related compounds (*4*). An excellent example is acyclic retinoid (ACR), a synthetic analog of vitamin A that is now under a randomized, double-blind, placebo-controlled phase III clinical trial in Japan to prevent the recurrence of HCC (*5*).

Vitamin K2 (VK2) is a fat-soluble vitamin essential for blood coagulation and calcium metabolism (*6*). It is known that the richest source of natural VK2 is the traditional Japanese food natto which is made of fermented soybeans (*7*). VK2 has received wide attentions for its health benefit such as for the prevention of osteoporosis (*8*). There has been continuing interest for the use of VK2 in the chemoprevention of HCC in Japan due to its long-term safety. In a small-scale clinical study originally designed to evaluate its preventive effect against osteoporosis, VK2 reduced the incidence of development of HCC in women with viral cirrhosis (*9*). A few other small-scale, controlled clinical trials also suggested the preventive role of VK2 on HCC recurrence (*10*, *11*). However, the efficacy of VK2 in suppressing HCC recurrence was not confirmed in a large-scale, double-blind, randomized, placebo-controlled clinical study enrolling 548 patients (*12*). Given these contradictory findings and the urgent need for chemoprevention of HCC, further study about the anti-HCC activity of VK2 is needed.

The present study was initiated to increase our understanding about the growth-suppressive effect of VK2 and its derivatives on HCC cells from a viewpoint of chemical structure. As we and the other groups have reported, ACR, a chemopreventive agent against HCC, has a vitamin A-like structure with three isoprene units in its carboxyl-terminated chain (*13*). ACR has been proven to have relatively low cytotoxicity (*14*); it selectively inhibits HCC cell growth but has a limited effect on normal liver cells (*15*). Here, we developed novel carboxylic derivatives of VK2 bearing isoprene structural units in their side chains and investigated their growth-suppression activity against HCC cells.

*To whom correspondence should be addressed.
E-mail: skojima@postman.riken.go.jp

MATERIALS AND METHODS

Chemicals. VK2 (menatetrenone; MK-4) and a transglutaminase activity inhibitor, cystamine (Cys), were purchased from Sigma-Aldrich (St. Louis, MO). A broad-spectrum caspase inhibitor, z-VAD-FMK (ZVAD) was obtained from R&D Systems (Abington, PA). Four carboxylic derivatives of VK2, namely SVK41, SVK24, SVK30 and SVK57, were freshly synthesized in Tokyo Medical and Dental University (*16*). Their chemical structures are summarized in Fig. 1. Ethanol (EtOH) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and used as the solvent of VK2 and its derivatives. EtOH solutions were further diluted into cell culture media for treatments. The final concentration of EtOH in media used as a control was 0.05% (vol/vol).

Cell culture. HCC cell lines, JHH7 and HepG2, were kindly supplied by Dr. Matsuura (Jikei University School of Medicine, Tokyo, Japan) (*17*). The normal human hepatocyte cell line (Hc) was purchased from Cell Systems (Kirkland, WA). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Wako Pure Chemical Industries, Ltd.) containing 10% fetal bovine serum (FBS, Mediatech, Herndon, VA), 100 U/mL penicillin/streptomycin and 2 mmol/L L-glutamine (Mediatech), and grown at 37°C in a humidified 5% CO_2 incubator. For chemical treatment, the cells were cultured in serum-free media containing chemicals at the appropriate concentrations.

Cell viability assay. Cell viability was determined using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Tokyo, Japan) in a plate reader (ARVO MX, Perkin Elmer Inc., Waltham, MA) as previously described (*15*).

Real-time RT-PCR. RNA was isolated from each cell culture using an RNeasy Kit (Qiagen, Valencia, CA) and cDNA was then synthesized using a PrimeScript RT Master Mix Kit (Takara, Otsu, Japan). Oligonucleotide primers were designed using OligoPerfect Designer software (Invitrogen, Carlsbad, CA). The sequences of the primers (5′ to 3′) are as follows: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward (CGACCACTTTGTCAAGCTCA) and reverse (AGGGGTCTACATGGCAACTG); Bcl-2 homologous antagonist/killer (Bak) forward (GGGTCTATGTTCCCCAGGAT) and reverse (GCAGGGGTAGAGTTGAGCAG). PCR reactions were performed using the Roche LightCycler® Real-Time PCR System (Roche Diagnostic Co., Ltd., Tokyo Japan) with SsoAdvanced™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA).

Small interfering RNA transfection. An siRNA targeting human Bak (sc-29786) and a control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Transfection was performed with 100 nM siRNAs using Lipofectamine 2000 (Life Technologies, Grand Island, NY) as previously described (*18*).

Statistical analysis. Quantitative data were expressed as the mean±SD from three independent experiments. The statistical significance of differences between values was assessed using a two-tailed Student's *t*-test. Values of $p<0.05$ were considered to indicate statistical significance.

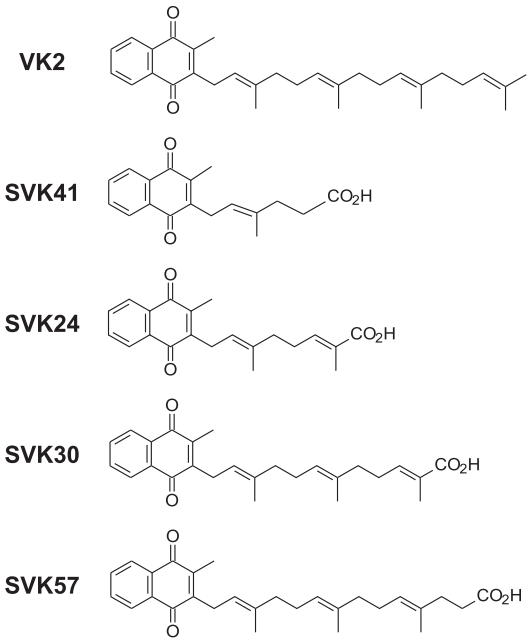

Fig. 1. Chemical structures of VK2 and its derivatives.

RESULTS

VK2 derivatives but not original VK2 suppressed JHH7 cell growth

Four carboxylic derivatives of VK2, SVK24, SVK30, SVK41 and SVK57, with different numbers of isoprene units in their side chains were synthesized (Fig. 1). The growth-suppression activity of these compounds was measured in an HCC cell line, JHH7 (Fig. 2). Of interest, no effect of VK2 was revealed at the high concentration of 50 μM for either 24 h or 48 h treatment (panel A, column 2). In contrast, carboxylic derivatives of VK2 such as SVK41, SVK24, SVK30 and SVK57 showed various extents of cell-growth inhibition in a time- and dose-dependent manner (panel A, columns 3–6 and panel B). These data suggested that the carboxylic group in the side chain is important for the HCC cell-growth inhibitory effect of VK2 derivatives. The antitumor activity of VK2 derivatives seemed to be dependent on their side chain lengths. The strongest effect was observed in SVK30, containing three isoprene units in its carboxylic-terminated side chain.

SVK24 selectively inhibited the growth of HCC cells but not Hc cells

The safety requirement for a chemopreventive agent is more stringent than for therapeutic agents due to the long-term administration to achieve a sufficient chemopreventive efficacy. To address this, we examined the growth-suppression activity of SVK24 in two HCC cell lines, JHH7 and HepG2, and a normal liver cell line, Hc. As the result, significant inhibitory effect of SVK24 was observed in HCC cells in a dose-dependent manner, while no effect was observed in normal hepatic cells (Fig. 3).

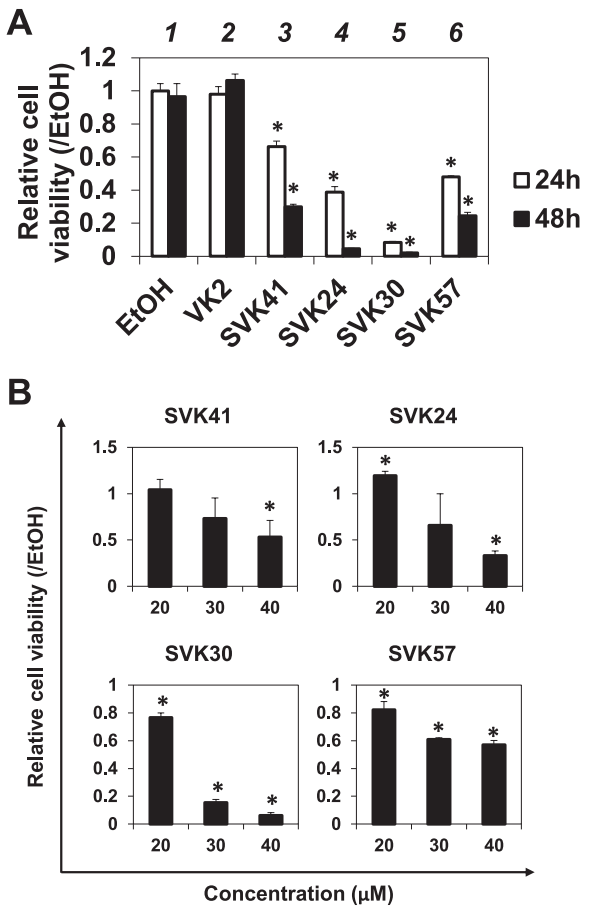

Fig. 2. Effect of VK2 and its derivatives on the proliferation of JHH7 cells. (A) Screening for the anti-HCC activities among VK2 and its derivatives, SVK41, SVK24, SVK30, and SVK57, after 24 h or 48 h treatment at the concentration of 50 μM. (B) Dose-dependent growth suppression of JHH7 cells treated with increasing concentrations of the VK2 derivatives SVK41, SVK24, SVK30, and SVK57 for 24 h. Cell viability was measured using the Cell Counting Kit-8 kit. Data were normalized to EtOH control and are expressed as the mean±SD (n=3). *p<0.05 compared with EtOH control.

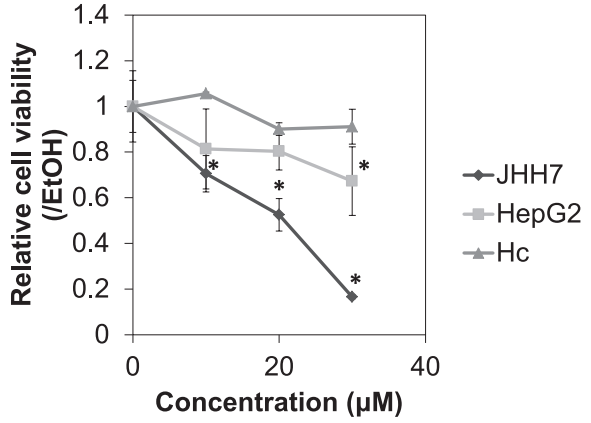

Fig. 3. Cancer-selective growth-suppressive effect of a VK2 derivative, SVK24. HCC cell lines, JHH7 and HepG2, as well as a normal hepatic cell line, Hc, were treated with increasing concentrations of SVK24 for 24 h. Data were normalized to EtOH control and are expressed as the mean±SD (n=3). *p<0.05 compared with EtOH control.

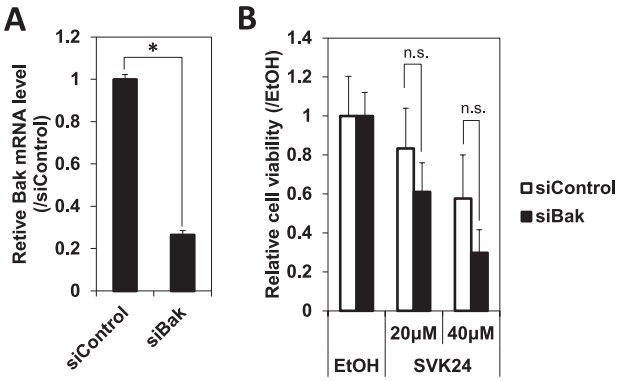

Fig. 4. Effect of Bak knockdown on SVK24-induced cell death in JHH7 cells. JHH7 cells were transfected with 100 nM siControl or siBak for 6 h in serum-free media using Lipofectamine 2000. Decreased Bak gene expression was confirmed 24 h after transfection (A). No significant effect of Bak knockdown was observed on SVK24-induced cell death in JHH7 cells (B). Data were normalized to siControl or EtOH control and are expressed as the mean±SD (n=3). *p<0.05 compared with siControl. n.s., not significant.

The effect of SVK24 was not mediated by a VK2 binding protein, Bak

To understand the molecular mechanism of the antitumor effect of VK2 derivatives, loss-of-function experiments using siRNA were examined in the growth-inhibitory effect of SVK24 in JHH7 cell cultures to uncover the role of Bak, a molecular target of VK2-induced apoptosis in HeLa cells (*19*). Treatment with an siRNA targeting human Bak (siBak) at 100 nM significantly reduced Bak mRNA expression (0.26-fold compared to siControl-treated cells) (Fig. 4A). However, Bak knockdown did not block the growth inhibitory effect of SVK24 in JHH7 cells and even worsened at both low (20 μM) and high concentrations (40 μM) (Fig. 4B).

Caspase/transglutaminase-dependent pathways

We have previously reported caspase- and transglutaminase-dependent pathways are associated with the apoptosis induced by ACR in HCC cells (*15*). Finally, we investigated whether VK2 derivatives also might suppress HCC cell growth in a caspase- and transglutaminase-dependent manner. To achieve this, we examined the effects of an irreversible general caspase inhibitor, ZVAD (*20*), and a transglutaminase activity inhibitor, Cys (*21*), on SVK24-induced cell death in JHH7 cells. Inhibited cell proliferation in JHH7 cells was significantly recovered after the addition of ZVAD in the presence of SVK24 at both low and high doses. Although the effect of Cys addition alone was only on the borderline of significance (p=0.06 in SVK24 treated cells at 40 μM), combined addition of ZVAD and Cys almost completely blocked the cell death induced by SVK24 in JHH7 cells (Fig. 5). Taken together, these findings suggested that

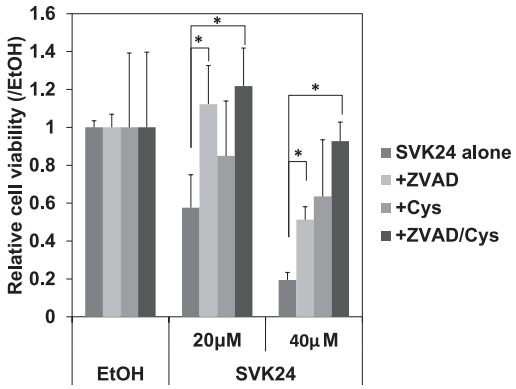

Fig. 5. Caspase and transglutaminase inhibitors blocked the growth suppression of JHH7 cells by SVK24. JHH7 cells were pretreated with 100 μM ZVAD or 250 μM Cys in serum-free media for 4 h, followed by treatment with SVK24 at the indicated concentrations in the absence or presence of 100 μM ZVAD or 250 μM Cys in serum- free media for 24 h. Data were normalized to EtOH control and are expressed as the mean±SD (n=3). *p<0.05 compared with SVK24 alone.

the inhibitory effect of the VK2 derivatives on HCC cell growth is at least in part via caspase/transglutaminase-dependent pathways.

DISCUSSION

The chemoprevention of HCC is one of the most challenging aspects of medical research (*13*). According to the concept of "field cancerization" (or "multicentric carcinogenesis") (*22*), the whole liver tissue is at high risk for possessing multiple, independent, and premalignant or latent malignant clones due to such causes as chronic infections with hepatitis B and C viruses and continued alcohol consumption. This hypothesis is supported by the exceedingly high recurrence rate of HCC; more than 70% of cases will recrudesce 5 y after primary curative treatments even at the early stage (*23*). Therefore, an effective and safe strategy of so-called cancer chemoprevention to block, reverse or delay the carcinogenic process of HCC is urgently needed (*24*). No adverse effects or hazards have ever been reported with supplementation with VK2 in its wide use in the therapy of bone loss (*24*). Furthermore, the potential ability of VK2 to induce the differentiation of human myeloid leukemia (*25*) and apoptosis of HCC cells (*26*) makes VK2 a promising chemopreventive agent against HCC. In this study, we demonstrated that new carboxylic derivatives of VK2 have cancer-selective growth suppression activities against HCC cells, providing candidate alternatives for current HCC prevention and treatment.

Given the inconsistency regarding the antitumor activity of VK2 against HCC in clinical trials (*9*, *10*, *12*), it is not surprising that VK2 itself failed to inhibit the growth of HCC cell line JHH7 in this study. In contrast, VK2 derivatives with isoprene residues in their carboxyl-terminated side chains time- and dose-dependently inhibited JHH7 cell growth. In accord with this, the growth suppression effect was not mediated by VK2 binding protein Bak, identified as a molecular target of VK2-induced apoptosis in the human cervical carcinoma cell line HeLa (*19*). It is considered that there is a differential molecular mechanism underlying the antitumor activity of VK2 derivatives observed in this study compared with that of VK2 in the other reports. Of particular interest, one of the VK2 derivatives, SVK41, is known as a VK2 metabolite, 2-methyl-3-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-naphthoquinone (*27*), suggesting a potential contribution of individual variants in VK2 metabolism to the inconsistent antitumor activities of VK2 in clinical trials.

ACR is another promising chemopreventive agent against the recurrence and development of HCC in patients after the surgical removal of primary tumors (*13*) and is currently undergoing a phase III clinical trial in Japan (5). Like ACR, SVK30 containing three isoprene residues in its carboxyl-terminated side chain exerted the strongest anti-proliferative activity in JHH7 cells. The structure of the carboxylic group and isoprene unit may play a common role in the induction of apoptosis in HCC cells. Furthermore, loss-of-function experiments using chemical inhibitors indicated that the growth-suppression effect of VK2 derivatives was mediated by caspase/transglutaminase-related signaling pathways, which are underlie the cancer-selective cell death induced by ACR in HCC cells (*15*). Indeed, synergistic growth inhibition by ACR and VK2 in combination has been reported in the human promyelocytic cell line HL60 (*28*) and HCC cell line Huh7 (*29*) by virtue of sharing a similar side chain structure. However, the IC50 values of VK2 derivatives are more than 20 μM (Fig. 2B), while that of ACR is around 10 μM (*15*). This difference is probably associated with the naphthoquinone ring structure of VK2 derivatives, which was identified as the Bak binding site of VK2 in HeLa cells (*19*). Although no statistically significant difference was observed, knockdown of Bak tended to enhance the growth suppression activity of SVK24 in JHH7 cells (Fig. 4B), suggesting a novel role of Bak to counteract the anti-HCC effect of the carboxylic derivatives of VK2.

In conclusion, the anti-proliferative effect of VK2 derivatives in HCC cells was investigated from the viewpoint of their chemical structure. Considering their cancer-selective anti-proliferative activities, in addition to hitherto known ACR, the carboxylic derivatives of VK2 that have isoprene structural units and a carboxyl-terminated side chain might prove to be potentially useful chemopreventive agents for the systemic treatment of HCC.

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REFERENCES

1) El-Serag HB. 2012. Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology* **142**: 1264–1273.
2) Zeng H, Zheng R, Guo Y, Zhang S, Zou X, Wang N, Zhang L, Tang J, Chen J, Wei K, Huang S, Wang J, Yu L, Zhao D, Song G, Chen J, Shen Y, Yang X, Gu X, Jin F, Li Q, Li Y, Ge H, Zhu F, Dong J, Guo G, Wu M, Du L, Sun X, He Y, Coleman MP, Baade P, Chen W, Yu XQ. 2014. Cancer survival in China, 2003–2005: A population-based study. *Int J Cancer* **136**: 1921–1930.
3) Steward WP, Brown K. 2013. Cancer chemoprevention: a rapidly evolving field. *Br J Cancer* **109**: 1–7.
4) Kakizoe T. 2003. Chemoprevention of cancer—focusing on clinical trials. *Jpn J Clin Oncol* **33**: 421–442.
5) Okita K, Izumi N, Ikeda K, Osaki Y, Numata K, Ikeda M, Kokudo N, Imanaka K, Nishiguchi S, Kondo S, Nishigaki Y, Shiomi S, Ueshima K, Isoda N, Karino Y, Kudo M, Tanaka K, Kaneko S, Moriwaki H, Makuuchi M, Okusaka T, Hayashi N, Ohashi Y, Kumada H; The Peretinoin Study Group. 2014. Survey of survival among patients with hepatitis C virus-related hepatocellular carcinoma treated with peretinoin, an acyclic retinoid, after the completion of a randomized, placebo-controlled trial. *J Gastroenterol* **50**: 667–674.
6) Beulens JW, Booth SL, van den Heuvel EG, Stoecklin E, Baka A, Vermeer C. 2013. The role of menaquinones (vitamin K(2)) in human health. *Br J Nutr* **110**: 1357–1368.
7) Kaneki M, Hodges SJ, Hosoi T, Fujiwara S, Lyons A, Crean SJ, Ishida N, Nakagawa M, Takechi M, Sano Y, Mizuno Y, Hoshino S, Miyao M, Inoue S, Horiki K, Shiraki M, Ouchi Y, Orimo H. 2001. Japanese fermented soybean food as the major determinant of the large geographic difference in circulating levels of vitamin K2: possible implications for hip-fracture risk. *Nutrition* **17**: 315–321.
8) Hamidi MS, Gajic-Veljanoski O, Cheung AM. 2013. Vitamin K and bone health. *J Clin Densitom* **16**: 409–413.
9) Habu D, Shiomi S, Tamori A, Takeda T, Tanaka T, Kubo S, Nishiguchi S. 2004. Role of vitamin K2 in the development of hepatocellular carcinoma in women with viral cirrhosis of the liver. *JAMA* **292**: 358–361.
10) Mizuta T, Ozaki I, Eguchi Y, Yasutake T, Kawazoe S, Fujimoto K, Yamamoto K. 2006. The effect of menatetrenone, a vitamin K2 analog, on disease recurrence and survival in patients with hepatocellular carcinoma after curative treatment: a pilot study. *Cancer* **106**: 867–872.
11) Kakizaki S, Sohara N, Sato K, Suzuki H, Yanagisawa M, Nakajima H, Takagi H, Naganuma A, Otsuka T, Takahashi H, Hamada T, Mori M. 2007. Preventive effects of vitamin K on recurrent disease in patients with hepatocellular carcinoma arising from hepatitis C viral infection. *J Gastroenterol Hepatol* **22**: 518–522.
12) Yoshida H, Shiratori Y, Kudo M, Shiina S, Mizuta T, Kojiro M, Yamamoto K, Koike Y, Saito K, Koyanagi N, Kawabe T, Kawazoe S, Kobashi H, Kasugai H, Osaki Y, Araki Y, Izumi N, Oka H, Tsuji K, Toyota J, Seki T, Osawa T, Masaki N, Ichinose M, Seike M, Ishikawa A, Ueno Y, Tagawa K, Kuromatsu R, Sakisaka S, Ikeda H, Kuroda H, Kokuryu H, Yamashita T, Sakaida I, Katamoto T, Kikuchi K, Nomoto M, Omata M. 2011. Effect of vitamin K2 on the recurrence of hepatocellular carcinoma. *Hepatology* **54**: 532–540.
13) Muto Y, Moriwaki H, Ninomiya M, Adachi S, Saito A, Takasaki KT, Tanaka T, Tsurumi K, Okuno M, Tomita E, Nakamura T, Kojima T. 1996. Prevention of second primary tumors by an acyclic retinoid, polyprenoic acid, in patients with hepatocellular carcinoma. Hepatoma Prevention Study Group. *N Engl J Med* **334**: 1561–1567.
14) Fukutomi Y, Omori M, Muto Y, Ninomiya M, Okuno M, Moriwaki H. 1990. Inhibitory effects of acyclic retinoid (polyprenoic acid) and its hydroxy derivative on cell growth and on secretion of alpha-fetoprotein in human hepatoma-derived cell line (PLC/PRF/5). *Jpn J Cancer Res* **81**: 1281–1285.
15) Tatsukawa H, Sano T, Fukaya Y, Ishibashi N, Watanabe M, Okuno M, Moriwaki H, Kojima S. 2011. Dual induction of caspase 3- and transglutaminase-dependent apoptosis by acyclic retinoid in hepatocellular carcinoma cells. *Mol Cancer* **10**: 4.
16) Fujii S, Shimizu A, Takeda N, Oguchi K, Katsurai T, Shirakawa H, Komai M, Kagechika H. 2015. Systematic synthesis and anti-inflammatory activity of ω-carboxylated menaquinone derivatives—Investigations on identified and putative vitamin K2 metabolites. *Bioorg Med Chem* **23**: 2344–2352.
17) Fujise K, Nagamori S, Hasumura S, Homma S, Sujino H, Matsuura T, Shimizu K, Niiya M, Kameda H, Fujita K. 1990. Integration of hepatitis B virus DNA into cells of six established human hepatocellular carcinoma cell lines. *Hepatogastroenterology* **37**: 457–460.
18) Qin XY, Wei F, Tanokura M, Ishibashi N, Shimizu M, Moriwaki H, Kojima S. 2013. The effect of acyclic retinoid on the metabolomic profiles of hepatocytes and hepatocellular carcinoma cells. *PLoS One* **8**: e82860.
19) Karasawa S, Azuma M, Kasama T, Sakamoto S, Kabe Y, Imai T, Yamaguchi Y, Miyazawa K, Handa H. 2013. Vitamin K2 covalently binds to Bak and induces Bak-mediated apoptosis. *Mol Pharmacol* **83**: 613–620.
20) Furukawa Y, Nishimura N, Furukawa Y, Satoh M, Endo H, Iwase S, Yamada H, Matsuda M, Kano Y, Nakamura M. 2002. Apaf-1 is a mediator of E2F-1-induced apoptosis. *J Biol Chem* **277**: 39760–39768.
21) Park MK, You HJ, Lee HJ, Kang JH, Oh SH, Kim SY, Lee CH. 2013. Transglutaminase-2 induces N-cadherin expression in TGF-beta1-induced epithelial mesenchymal transition via c-Jun-N-terminal kinase activation by protein phosphatase 2A down-regulation. *Eur J Cancer* **49**: 1692–1705.
22) Ki Hong W, Lippman SM, Hittelman WN, Lotan R. 1995. Retinoid chemoprevention of aerodigestive cancer: from basic research to the clinic. *Clin Cancer Res* **1**: 677–686.
23) Tung-Ping Poon R, Fan ST, Wong J. 2000. Risk factors, prevention, and management of postoperative recurrence after resection of hepatocellular carcinoma. *Ann Surg* **232**: 10–24.
24) Weber P. 2001. Vitamin K and bone health. *Nutrition* **17**: 880–887.
25) Yaguchi M, Miyazawa K, Katagiri T, Nishimaki J, Kizaki M, Tohyama K, Toyama K. 1997. Vitamin K2 and its derivatives induce apoptosis in leukemia cells and enhance the effect of all-trans retinoic acid. *Leukemia*

11: 779–787.

26) Otsuka M, Kato N, Shao RX, Hoshida Y, Ijichi H, Koike Y, Taniguchi H, Moriyama M, Shiratori Y, Kawabe T, Omata M. 2004. Vitamin K2 inhibits the growth and invasiveness of hepatocellular carcinoma cells via protein kinase A activation. *Hepatology* **40**: 243–251.

27) Shearer MJ, Newman P. 2008. Metabolism and cell biology of vitamin K. *Thromb Haemost* **100**: 530–547.

28) Kitagawa J, Hara T, Tsurumi H, Ninomiya S, Ogawa K, Adachi S, Kanemura N, Kasahara S, Shimizu M, Moriwaki H. 2011. Synergistic growth inhibition in HL-60 cells by the combination of acyclic retinoid and vitamin K2. *J Cancer Res Clin Oncol* **137**: 779–787.

29) Kanamori T, Shimizu M, Okuno M, Matsushima-Nishiwaki R, Tsurumi H, Kojima S, Moriwaki M. 2007. Synergistic growth inhibition by acyclic retinoid and vitamin K2 in human hepatocellular carcinoma cells. *Cancer Sci* **98**: 431–437.