



Amelioration of carcinogenesis and tumor growth in the rat liver by combination of vitamin K₂ and angiotensin-converting enzyme inhibitor via anti-angiogenic activities

HITOSHI YOSHIJI¹, SHIGEKI KURIYAMA², RYUICHI NOGUCHI¹, JUNICHI YOSHII¹, YASUHIDE IKENAKA¹, KOJI YANASE¹, TADASHI NAMISAKI¹, MITSUTERU KITADE¹, MASAHARU YAMAZAKI¹, TAKEMI AKAHANE¹, KIYOSHI ASADA¹, TATSUHITO TSUJIMOTO¹, MASAHITO UEMURA¹ and HIROSHI FUKUI¹

¹Third Department of Internal Medicine, Nara Medical University, Nara; ²Third Department of Internal Medicine, Kagawa University School of Medicine, Kagawa, Japan

Received July 4, 2005; Accepted August 19, 2005

Abstract. Recent studies have revealed that angiogenesis plays a pivotal role in carcinogenesis and tumor growth. We previously reported that the clinically used vitamin K₂ (VK) and angiotensin-converting enzyme inhibitor (ACE-I) exerted potent anti-angiogenic activities. The aim of our current study was to examine the combination effect of VK and ACE-I on hepatocarcinogenesis induced by diethyl-nitrosamine, and orthotopic hepatocellular carcinoma (HCC) growth in rats. When used individually, both VK and ACE-I at clinically comparable low doses exerted significant inhibitory effects on tumor development in the liver. A combination treatment of VK and ACE-I showed a more potent suppressive effect against hepatocarcinogenesis. Neovascularization increased during hepatocarcinogenesis, and VK and ACE-I significantly attenuated angiogenesis in the tumor. In orthotopic HCC transplantation, VK and ACE-I also showed marked suppressive effects against HCC development similar to those against hepatocarcinogenesis. In both experiments, the suppressive effects of VK and ACE-I against angiogenesis were similar in magnitude to their inhibitory effects against hepatocarcinogenesis and orthotopic HCC development. In the orthotopic model, VK and ACE-I treatment resulted in a marked increase of apoptosis in the tumor, whereas tumor cell proliferation itself was not altered. Since both VK and ACE-I are widely used in clinical practice without serious side effects, this combination

therapy may be an effective new therapeutic strategy against hepatocarcinogenesis and HCC growth in the future.

Introduction

Tumor growth, including hepatocellular carcinoma (HCC), is angiogenesis-dependent. Any solid tumor that has not acquired its own blood supply cannot grow to more than only a few millimeters in size (1,2). Therapies targeting the tumor vessels have proven successful for cancer treatment in experimental models (3). We previously reported that angiogenesis plays a pivotal role in murine HCC development, and suppression of the angiogenic-signaling pathway markedly attenuated tumor growth (4-6). In addition to tumor growth, studies have demonstrated that angiogenesis begins at an early stage when the tumor consists of just 100-300 cells (7). Moreover, several reports have shown that angiogenesis is involved in the early carcinogenesis step (8-10). A study on endothelial cell markers in dysplastic lesions of the liver has suggested that alterations in the hepatic microcirculation occur at an early stage of liver carcinogenesis in HCC (11). We previously proved that angiogenesis played a pivotal role in murine hepatocarcinogenesis (12).

HCC is one of the most common malignancies in the world, and its prognosis is still poor since it develops in conjunction with chronic liver diseases, such as liver cirrhosis (13). Radical operation is the only curative modality for HCC, but is appropriate for a minority of patients due to limited hepatic reserves. Therefore, various palliative therapeutic modalities, such as transarterial embolization (TAE), percutaneous intratumoral ethanol injection (PEIT) and orthotopic transplantation, have been employed. However, there is still no satisfactory treatment for HCC. An anti-angiogenic therapy would be a fruitful approach against HCC. To date, several new anti-angiogenic agents are under investigation around the world. Although some of these agents are now in clinical trials at limited institutes, no agents are widely or currently available in clinical practice (14). Under the concept of anti-angiogenesis therapy, long-term

Correspondence to: Dr Hitoshi Yoshiji, Third Department of Internal Medicine, Nara Medical University, Shijo-cho 840, Kashihara, Nara 634-8522, Japan
E-mail: yoshijih@narmed-u.ac.jp

Key words: angiogenesis, vitamin K₂, angiotensin-converting enzyme, renin-angiotensin system, hepatocarcinogenesis, hepatocellular carcinoma

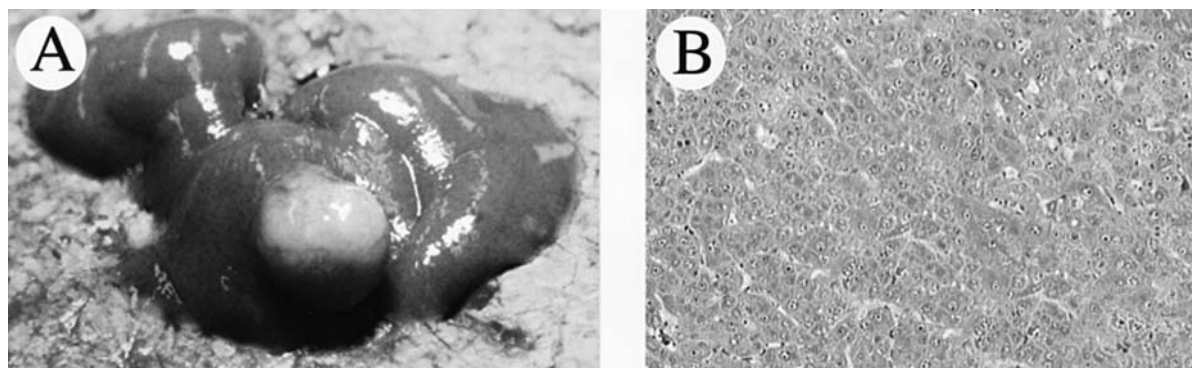


Figure 1. Typical macroscopic and microscopic features of HCC in the DEN-treated liver. (A) Typical multiple hepatic nodules in the DEN-treated liver are whitish with relatively clear margins, and neovascularization can be seen on the tumor surface. (B) HCC displayed a trabecular pattern with cords of more than one hepatocyte in thickness. Original magnification, x200.

administration is required to examine the compound toxicity. A potential alternative strategy may be the use of drugs with anti-angiogenic activity, which are available in oral form and currently used for the treatment of different diseases.

Some of the clinically available compounds, such as thalidomide and penicillamine, have been shown to possess anti-angiogenic activity, and are presently used in clinical trials (14). However, long-term administration of these agents sometimes leads to severe side effects, such as bone marrow suppression. We previously reported that the clinically used angiotensin-converting enzyme inhibitor (ACE-I), perindopril (PE), possesses strong anti-angiogenic activity, and inhibited murine HCC growth at clinically comparable low doses (15).

A Japanese clinical study has revealed that vitamin K₂ (VK) exerted a suppressive effect against the development of HCC in women with viral hepatic cirrhosis (16). We have previously reported that VK also showed anti-angiogenic activity, and the combination treatment of VK and ACE-I at clinically comparable low doses showed a marked suppressive effect against the development of liver enzyme-altered preneoplastic lesions in rats via angiogenesis suppression (17).

In the current study, we examined whether this combination regimen really suppresses HCC carcinogenesis rather than the development of preneoplastic lesions. We also elucidated that this regimen inhibited HCC growth in the liver. Since orthotopic transplantation is more relevant to a clinical situation than the subcutaneous tumor (18), we employed an orthotopic experimental model. We sought to clarify the possible mechanism, especially in conjunction with the alteration of neovascularization.

Materials and methods

Compounds and animal treatment. ACE-I (PE) and VK were generously supplied by Daiichi Pharmaceutical Co (Tokyo, Japan), and Eisai Inc. (Tokyo, Japan), respectively. Male BALB/c mice, aged 6 weeks, were purchased from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan) and housed in stainless-steel, mesh cages under controlled conditions of temperature (23±3°C) and relative humidity (50±20%), with 10-15 air changes per hour and light illumination for 12 h a day. The animals were allowed access to food and tap water

ad libitum throughout the acclimatization and experimental periods. In the carcinogenesis experiment, the experimental period was 36 weeks. The mice were divided into 5 groups (n=15 each). The mice in groups 1 to 4 (G1 to G4) received an intraperitoneal (i.p.) injection of 75 mg/kg of diethylnitrosamine (DEN) weekly for 3 weeks, then 100 mg/kg of DEN for 3 weeks followed by phenobarbital (PB) mixed with each diet at a concentration of 0.05% from week 6 to the final sacrifice, as described previously (19,20). The mice in G1 did not receive any additional treatment, and were considered the control group. The mice in G2 and G3 received 3 mg/kg/day of VK and 2 mg/kg/day of ACE-I by daily gavage, respectively. It has been reported that the doses of these agents are almost comparable to those used in clinical practice (15,21). The combination treatment of VK and ACE-I group was designated as G4. The mice, which received phosphate-buffered saline (PBS) instead of DEN, were examined as the negative control group (G5). All mice were anesthetized, the thoracic cavity was opened, and blood samples were withdrawn via cardiac puncture. The entire livers from randomly chosen mice in each group (n=10) were removed, weighed and cut into 2-mm-thick strips to examine the presence of macroscopically visible lesions. All macroscopic lesions were described, mapped, and quantitated by size, as described previously (22). For the orthotopic HCC transplantation experiment, 1×10⁶ of BNL-HCC cells were directly injected into the liver, as described previously (4) (n=7 for each group). The cells were suspended in 10 μl of PBS and implanted directly under the capsule of the left lateral hepatic lobe under direct visualization, by means of a 10-μl Hamilton syringe with a 26-gauge needle over a period of 2-3 min. Treatments with VK and ACE-I were the same as those in the carcinogenesis experiment. The mice were sacrificed 14 days after injection and examined macro- and microscopically for HCC development in the liver. All animal procedures were performed according to standard protocol and in accordance with the standard recommendations for the proper care and use of laboratory animals.

mRNA expression of CD31. The mRNA expression of CD-31, which is used widely as a marker of neovascularization, was evaluated by real-time PCR as described previously (23). In both experiments, the macroscopically visible tumor lesions and adjacent tissues were separated, and half of the tumor

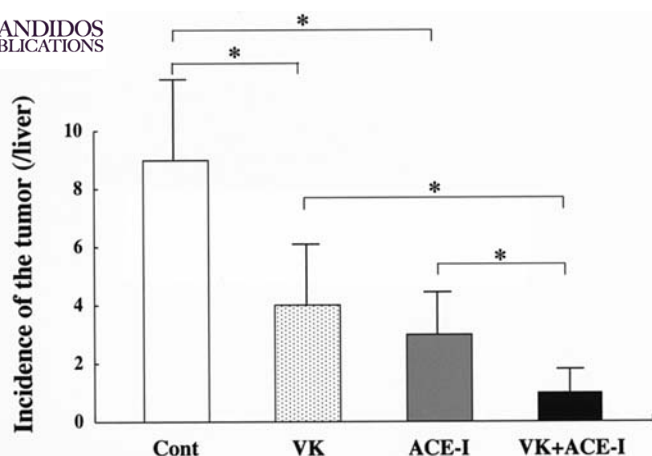


Figure 2. Effects of VK and ACE-I on microscopic development of the liver tumor. The incidence of HCC in the liver was significantly inhibited by treatments with VK and ACE-I. The combination treatment of VK and ACE-I exerted a more potent inhibitory effect compared with single agent treatment. Cont, control DEN-treated mice; VK and ACE-I, VK- and ACE-I-treated mice; VK+ACE-I, VK and ACE-I combination-treated group. The data represent means \pm SD (n=10). *Statistically significant differences between the indicated groups (p<0.01).

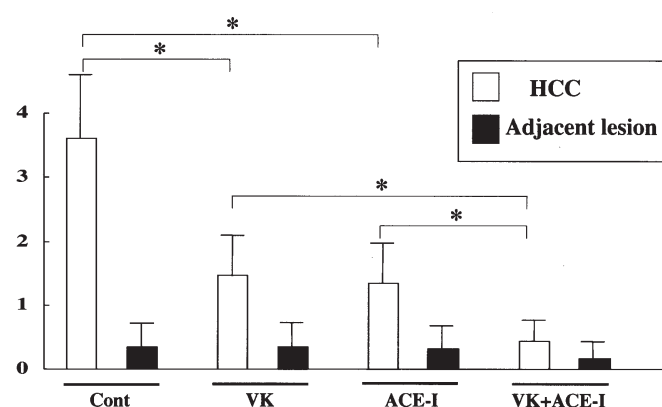


Figure 3. CD31 mRNA expression in the DEN-treated liver. The mRNA expression was measured by real-time PCR as described in Materials and methods. The mRNA expression of CD31 was significantly higher compared with the surrounding adjacent non-malignant tissues. Treatment with either VK or ACE-I markedly suppressed CD31 expression compared with the control group. The combination treatment of VK and ACE-I showed a more potent inhibitory effect. Cont, control DEN-treated mice; VK and ACE-I, VK- and ACE-I-treated mice; VK+ACE-I, VK and ACE-I combination-treated group. The data represent means \pm SD (n=10). *Statistically significant differences between the indicated groups (p<0.01).

tissue was immediately snap-frozen for RNA extraction (n=5 in each experimental group). We used the same size tumor to avoid the necrotic effect of hypoxic conditions, as described previously (23). Real-time PCR was performed with the ABI Prism 7700 Sequence Detection System (PE-I Applied Biosystems, Foster City, CA, USA) according to the manufacturer's manual. Relative quantitation of the gene expression was performed as described in the manual by using glyceraldehyde-3-phosphate dehydrogenase as an internal control.

Immunohistochemistry. Apoptosis in the tumor was detected with DNA fragmentation products that were stained with

in situ 3' end labeling [terminal deoxynucleotidyl transferase-mediated dUTP nick-labeling (TUNEL)]. The *in vivo* tumor cell proliferation was evaluated by quantification of the cells positive for the proliferative cell nuclear antigen (PCNA). Immunohistochemistry for PCNA and TUNEL was performed as described previously (24). The PCNA- and TUNEL-positive cells were enumerated in the VK- and ACE-I-treated and control groups, using a light microscope. In each tumor, the positive cells in 30 high-power fields at a magnification of x400 were examined.

Statistical analysis. To assess the statistical significance of intergroup differences in the quantitative data, Bonferroni's multiple comparison test was used after one-way analysis of variance (ANOVA). This was followed by Bartlett's test to determine the homology of variance.

Results

Effects of VK and ACE-I against hepatocarcinogenesis. We first examined the effects of VK and ACE-I against hepatocarcinogenesis. Fig. 1A shows the typical pale whitish macroscopic appearance of hepatic lesions. Treatment with DEN resulted in a marked development of hepatic nodules. Histological examination of the hepatic nodules displayed HCC (Fig. 1B), which was shown primarily as a trabecular pattern with cords of more than one hepatocyte in thickness. The incidence of HCC in the liver was significantly inhibited by treatments with VK and ACE-I. The combination treatment of VK and ACE-I exerted a more potent inhibitory effect compared with the single agent treatments (Fig. 2). No evidence of tumor development could be found in the PBS-treated group (G5; data not shown).

Effects of VK and ACE-I on neovascularization in the liver. We next examined the mRNA expression of CD31 in the liver. The mRNA expression of CD31 in HCC was significantly increased compared with the surrounding adjacent lesions (Fig. 3). Treatment with VK and ACE-I significantly suppressed CD31 expression compared to the control group (p<0.01). The combination treatment using both agents nearly abolished neovascularization in the tumor. Noteworthy was the finding that the suppression of angiogenesis by treatment with VK and ACE-I was of similar magnitude to the inhibition of HCC development.

Orthotopic HCC transplantation. We also examined whether the combination treatment of VK and ACE-I could inhibit HCC growth or not. As shown in Fig. 4, similar to their effects against hepatocarcinogenesis, both VK and ACE-I significantly suppressed HCC growth in the liver. Combination treatment with both agents revealed further inhibition compared with VK or ACE-I alone. Neither tumor invasion into other organs nor distant metastasis was observed at the time of sacrifice (data not shown). Neovascularization in the tumor was markedly attenuated by treatment with VK and ACE-I at a magnitude similar to that of tumor growth inhibition (Table I).

Immunohistochemistry. To elucidate the possible mechanism, we examined whether the tumor inhibition by VK and ACE-I

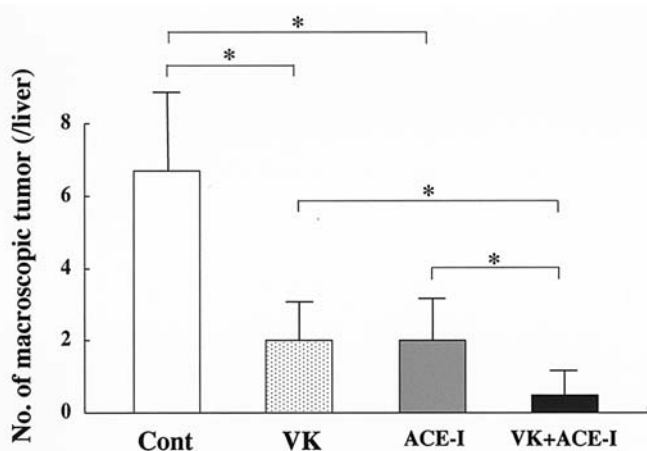


Figure 4. Effects of VK and ACE-I on orthotopically transplanted HCC growth. The number of macroscopic tumors in the livers from VK and ACE-I-treated groups was significantly suppressed compared with the control group. Combination treatment with both agents revealed further inhibition in comparison with VK or ACE-I alone. Cont, control mice; VK and ACE-I, VK- and ACE-I-treated mice; VK+ACE-I, VK and ACE-I combination-treated group. The data represent means \pm SD (n=7). *Statistically significant differences between the indicated groups ($p < 0.01$).

was reflected in tumor cell proliferation and apoptosis, via immunohistochemical analysis of PCNA and TUNEL. As shown in Table I, the PCNA-positive cell index was apparently not altered by either VK or ACE-I treatment. On the contrary, the number of TUNEL-positive cells was significantly increased by treatment with VK and ACE-I compared with the control group. The incidence of apoptosis in the tumor almost corresponded to the effect of tumor growth inhibition, suggesting that VK and ACE-I resulted in tumor inhibition by augmentation of apoptosis in the tumor, but not via suppression of tumor cell proliferation in the current study.

Discussion

It is now widely recognized that angiogenesis plays a pivotal role in the development of solid tumors, including HCC (2,25,26). Any tumor mass in excess of a few cubic millimeters depends on the formation of a vascular network that provides the growing tumor with oxygen and essential nutrients. Until recently, it had been believed that angiogenesis starts at a relatively late stage when the tumor reaches a size of several hundred microns to 1 mm in diameter or contains roughly

10^5 - 10^6 cells (27). It has been demonstrated that angiogenesis begins at an early stage when the tumor contains just 100-300 cells (7). Several reports have also shown that angiogenesis is involved in early carcinogenesis (8-10).

Anti-angiogenic therapy is under investigation all over the world, and includes the use of gene therapy, anti-angiogenic recombinant proteins, monoclonal antibodies, and various drugs (14). However, some studies have suggested that a treatment using a single anti-angiogenic agent may not be sufficient to completely inhibit tumor angiogenesis (28,29). The use of anti-angiogenic agents, such as monotherapy, in treating patients with advanced cancer has not yet shown significant efficacy (2,14). The limitations of anti-angiogenic monotherapy in this setting were in fact predicted by pre-clinical studies on the angiogenesis inhibitors endostatin and angiostatin. It has been reported that combination treatment with anti-angiogenic agents, such as endostatin and angiostatin, exerted a synergistic inhibitory effect on tumor development and angiogenesis (14,30). We previously reported that the combination treatment of VK and ACE-I at clinically comparable low doses attenuated preneoplastic lesion development in the liver, in association with the suppression of neovascularization (21). In the current study, we performed a long-term experiment and found that this combination regimen suppressed HCC development and exerted a more potent inhibitory effect compared with either single agent. Furthermore, we revealed that VK and ACE-I attenuated orthotopic HCC growth.

It has been shown that treatment with anti-angiogenic agents induced a marked increase of apoptosis in the tumor, but did not alter tumor cell proliferation itself (31). In this study, immunohistochemical analysis by TUNEL assay revealed that VK and ACE-I significantly increased apoptosis in the tumor, whereas tumor cell proliferation in the tumor was not altered by treatment with VK and ACE-I as evaluated by PCNA immunohistochemistry. It has been reported that a high dose of VK could suppress the proliferation of HCC cells via protein kinase A inhibition *in vitro* (32). However, we previously observed that neither VK nor ACE-I altered the proliferation of HCC cells at a clinically comparable low dose. On the contrary, both agents significantly suppressed endothelial cell (EC) tubular formation, even at low doses (15,17). These findings, taken together, suggest that VK and ACE-I reduced tumor growth by inhibiting EC tubular formation, which in turn may stimulate the induction of apoptosis of tumor cells, rather than direct inhibition of tumor

Table I. Effect of VK and/or ACE-I on CD31 mRNA, proliferation and apoptosis positive cells in the tumors.

	Control	VK	ACE-I	VK+ACE-I
CD31 mRNA	4.23 ^a	1.21	1.18	0.52
Proliferation	17.10 \pm 7.42 ^b	14.80 \pm 8.86	15.30 \pm 8.24	15.70 \pm 7.89
Apoptosis	1.05 \pm 0.88	4.65 \pm 2.24 ^c	5.87 \pm 3.69 ^c	9.22 \pm 4.34 ^{c,d}

^aGene expression presented after normalization with the glyceraldehyde-3-phosphate dehydrogenase internal control. ^bData represent the mean \pm SD (n=7 in each group). ^{c,d}Statistically significant difference compared with the control group ($p < 0.01$) and VK or ACE-I-treated group ($p < 0.01$), respectively.



eration. Nevertheless, it was important to determine apoptosis was observed mainly in the EC or HCC cells, but no precise answers are available at this time. Although we performed a double immunohistochemical analysis with CD31 and TUNEL on more than one occasion, we failed to obtain a good result as the background was intense and interpretation was difficult (data not shown). It has been suggested that apoptosis of EC occurred first, which led to secondary apoptosis of tumor cells with anti-angiogenic agents (33,34). As described above, we previously found that neither VK nor ACE-I affected the *in vitro* proliferation of BNL-HCC cells. These findings suggest that VK and ACE-I first induced EC apoptosis, and this might induce secondary apoptosis of the tumor cells. Further studies are required to elucidate the exact mechanisms involved.

In summary, we have shown that the combination treatment of VK and ACE-I significantly inhibited hepatocarcinogenesis and HCC growth and suppressed angiogenesis. Noteworthy was the fact that these inhibitory effects were achieved at clinically comparable low doses. Since both agents are widely used in clinical practice, this combination regimen may represent a potential new strategy against HCC in the future.

References

- Carmeliet P: Angiogenesis in health and disease. *Nat Med* 9: 653-660, 2003
- Carmeliet P and Jain RK: Angiogenesis in cancer and other diseases. *Nature* 407: 249-257, 2000.
- Saaristo A, Karpanen T and Alitalo K: Mechanisms of angiogenesis and their use in the inhibition of tumor growth and metastasis. *Oncogene* 19: 6122-6129, 2000.
- Yoshiji H, Kuriyama S, Hicklin DJ, *et al*: KDR/Flk-1 is a major regulator of vascular endothelial growth factor- induced tumor development and angiogenesis in murine hepatocellular carcinoma cells. *Hepatology* 30: 1179-1186, 1999.
- Yoshiji H, Kuriyama S, Yoshii J, *et al*: Involvement of the vascular endothelial growth factor receptor-1 in murine hepatocellular carcinoma development. *J Hepatol* 41: 97-103, 2004.
- Yoshiji H, Kuriyama S, Yoshii J, *et al*: Vascular endothelial growth factor tightly regulates *in vivo* development of murine hepatocellular carcinoma cells. *Hepatology* 28: 1489-1496, 1998.
- Li CY, Shan S, Huang Q, *et al*: Initial stages of tumor cell-induced angiogenesis: evaluation via skin window chambers in rodent models. *J Natl Cancer Inst* 92: 143-147, 2000.
- Bergers G, Javaherian K, Lo KM, Folkman J and Hanahan D: Effects of angiogenesis inhibitors on multistage carcinogenesis in mice. *Science* 284: 808-812, 1999.
- Brandvold KA, Neiman P and Ruddell A: Angiogenesis is an early event in the generation of myc-induced lymphomas. *Oncogene* 19: 2780-2785, 2000.
- Boltontrade MF, Stern MC, Binder RL, Zenklusen JC, Conti CJ, and Gimenez-Conti IB: Angiogenesis is an early event in the development of chemically induced skin tumors. *Carcinogenesis* 19: 2107-2113, 1998.
- Frachon S, Gouysse G, Dumorti J, *et al*: Endothelial cell marker expression in dysplastic lesions of the liver: an immunohistochemical study. *J Hepatol* 34: 850-857, 2001.
- Yoshiji H, Kuriyama S, Yoshii J, *et al*: Halting the interaction between vascular endothelial growth factor and its receptors attenuates liver carcinogenesis in mice. *Hepatology* 39: 1517-1524, 2004.
- Befeler AS and Di Bisceglie AM: Hepatocellular carcinoma: diagnosis and treatment. *Gastroenterology* 122: 1609-1619, 2002.
- Kerbel RS: Clinical trials of antiangiogenic drugs: opportunities, problems, and assessment of initial results. *J Clin Oncol* 19: 45S-51S, 2001.
- Yoshiji H, Kuriyama S, Kawata M, *et al*: The angiotensin-converting enzyme inhibitor perindopril suppresses tumor growth and angiogenesis: possible role of the vascular endothelial growth factor. *Clin Cancer Res* 7: 1073-1078, 2001.
- Habu D, Shiomi S, Tamori A, *et al*: Role of Vitamin K₂ in the development of hepatocellular carcinoma in women with viral cirrhosis of the liver. *JAMA* 292: 358-361, 2004.
- Yoshiji H, Kuriyama S, Noguchi R, *et al*: Combination of vitamin K(2) and the angiotensin-converting enzyme inhibitor, perindopril, attenuates the liver enzyme-altered preneoplastic lesions in rats via angiogenesis suppression. *J Hepatol* 42: 687-693, 2005.
- Kuriyama S, Masui K, Kikukawa M, *et al*: Complete cure of established murine hepatocellular carcinoma is achievable by repeated injections of retroviruses carrying the herpes simplex virus thymidine kinase gene. *Gene Ther* 6: 525-533, 1999.
- Yoshiji H, Nakae D, Kinugasa T, *et al*: Inhibitory effect of dietary iron deficiency on the induction of putative preneoplastic foci in rat liver initiated with diethylnitrosamine and promoted by phenobarbital. *Br J Cancer* 64: 839-842, 1991.
- Shiota G, Harada K, Ishida M, *et al*: Inhibition of hepatocellular carcinoma by glycyrrhizin in diethylnitrosamine-treated mice. *Carcinogenesis* 20: 59-63, 1999.
- Yoshiji H, Kuriyama S, Noguchi R, *et al*: Combination of interferon-beta and angiotensin-converting enzyme inhibitor, perindopril, attenuates the murine liver fibrosis development. *Liver Int* 25: 153-161, 2005.
- Moser GJ, Wong BA, Wolf DC, Fransson-Steen RL and Goldsworthy TL: Methyl tertiary butyl ether lacks tumor-promoting activity in N-nitrosodiethylamine-initiated B6C3F1 female mouse liver. *Carcinogenesis* 17: 2753-2761, 1996.
- Yoshiji H, Kuriyama S, Yoshii J, *et al*: Synergistic effect of basic fibroblast growth factor and vascular endothelial growth factor in murine hepatocellular carcinoma. *Hepatology* 35: 834-842, 2002.
- Yoshiji H, Harris SR, Raso E, *et al*: Mammary carcinoma cells over-expressing tissue inhibitor of metalloproteinases-1 show enhanced vascular endothelial growth factor expression. *Int J Cancer* 75: 81-87, 1998.
- Carmeliet P: Mechanisms of angiogenesis and arteriogenesis. *Nat Med* 6: 389-395, 2000.
- Harris SR and Thorgeirsson UP: Tumor angiogenesis: biology and therapeutic prospects. *In Vivo* 12: 563-570, 1998.
- Folkman J: Tumor angiogenesis: therapeutic implications. *N Engl J Med* 285: 1182-1186, 1971.
- Kerbel RS and Kamen BA: The anti-angiogenic basis of metronomic chemotherapy. *Nat Rev Cancer* 4: 423-436, 2004.
- Davis DW, Inoue K, Dinney CP, Hicklin DJ, Abbruzzese JL and McConkey DJ: Regional effects of an antivascular endothelial growth factor receptor monoclonal antibody on receptor phosphorylation and apoptosis in human 253J B-V bladder cancer xenografts. *Cancer Res* 64: 4601-4610, 2004.
- Scappaticci FA, Smith R, Pathak A, *et al*: Combination angiostatin and endostatin gene transfer induces synergistic antiangiogenic activity *in vitro* and antitumor efficacy in leukemia and solid tumors in mice. *Mol Ther* 3: 186-196, 2001.
- Yoshii J, Yoshiji H, Kuriyama S, *et al*: The copper-chelating agent, trientine, suppresses tumor development and angiogenesis in the murine hepatocellular carcinoma cells. *Int J Cancer* 94: 768-773, 2001.
- Otsuka M, Kato N, Shao RX, *et al*: Vitamin K₂ inhibits the growth and invasiveness of hepatocellular carcinoma cells via protein kinase A activation. *Hepatology* 40: 243-251, 2004.
- Browder T, Butterfield CE, Kraling BM, *et al*: Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. *Cancer Res* 60: 1878-1886, 2000.
- Hlatky L, Hahnfeldt P and Folkman J: Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us. *J Natl Cancer Inst* 94: 883-893, 2002.