Cytochrome P450 2E1 increases the sensitivity of hepatoma cells to vitamin K2

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Abstract. Although vitamin K2 (VK2) exhibits inhibitory effects on the viability of hepatoma cells, hepatoma cells are insensitive to VK2. Therefore, this investigation is an attempt to enhance the sensitivity of hepatoma cells to VK2. Our results showed that VK2 acted synergistically with ethanol (EtOH) to inhibit the viability of Smmc-7721 cells, mainly because cytochrome P450 2E1 (CYP2E1) was activated by EtOH. The synergistic effect of VK2 and EtOH was also observed in QGY-7703 cells, which also express CYP2E1. However, in HepG2 cells, which do not express CYP2E1, the synergistic effect of VK2 and EtOH was not observed. In addition, we demonstrated that CYP2E1 could be induced by VK2 via both post-transcriptional and transcriptional mechanisms. These results suggest that induction of CYP2E1 can enhance the inhibitory effect of VK2 on the viability of hepatoma cells. CYP2E1 may be an attractive target for enhanced antitumor effects of VK2 in hepatocellular carcinoma treatment.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most important cause of cancer related death in men (1,2). Each year the growing number of new HCC cases being diagnosed is nearly equal to the number of deaths from this disease (1,2). In addition, although for HCC treatment variable degree of benefit has been observed by use of chemotherapeutic agents such as interferon (3,4), adverse effects such as bone marrow suppression after long-term administration limit their usefulness (5). Therefore, alternative drugs are needed to improve the prognosis and survival of patients.

Vitamin K2 (VK2), is used clinically as an activator of homeostasis and an inhibitor of osteoporosis (6,7), recently has been reported to inhibit the growth of hepatoma cells (8-10) and in HCC patients decrease serum levels of des-gamma-carboxy prothrombin (DCP), a significant predisposing factor for the development of portal venous invasion of HCC (11,12). Furthermore, VK2 is a safe medicine without prominent adverse effects such as bone marrow suppression after long-term administration (13).

Although VK2 has potential for the treatment of liver cancer, it has also been reported that hepatoma is insensitive to VK2 (14). VK2 administration alone was reported to be insufficient to prevent HCC recurrence in clinic (15,16). For more effective use of VK2 in HCC treatment, it is necessary to increase the sensitivity of hepatoma to VK2.

Cytochrome P450 2E1 (CYP2E1), as an enzyme from CYP2 family, known to regulate cytokine signaling, antigen presentation, and macromolecular degradation, all of which are crucial to liver cell function and viability (17). Furthermore, previous findings indicated that CYP2E1 synergized and increased the susceptibility of hepatocytes to different chemicals (18,19). Therefore, we speculated that CYP2E1 may play an important role in the enhanced susceptibility of hepatomas to VK2. In the present study, we demonstrated that CYP2E1 efficiently enhanced the inhibitory effect of VK2 on the viability of hepatoma cells. Furthermore, we showed that CYP2E1 was regulated by VK2 via both post-transcriptional and transcriptional mechanisms in hepatoma cells.

Materials and methods

Materials. VK2 was purchased from Sigma, St. Louis, MO, USA (no. V9378). It has also been termed as menatetrenone (MK-4), one of nine forms of vitamin K2, which is ubiquitously present in extrahepatic tissues.

Cell culture. Human hepatocellular carcinoma Smmc-7721, QGY-7703 and HepG2 cells were kindly provided by Prof. S. Ge, Shanghai Jiaotong University, School of Medicine,

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Shanghai, China. All cells were checked with the list of known mis-identified cell lines available from the International Cell Line Authentication Committee (http://iclac.org/databases/cross-contaminations) and confirmed that they are not mis-identified or contaminated. All cells were cultured in RPMI-1640 medium (Thermo Scientific Hyclone, Logan, UT, USA) supplemented with 12% fetal bovine serum (FBS; Thermo Scientific Hyclone) in a humidified atmosphere with 5% CO_2 at 37˚C.

Viability assays. Cells/well ($5x10^3$) were seeded into 96-well plates and treated with VK2 (Sigma) as indicated in the result section. Cell viability was measured by MTT assay. The optical density (OD) of each well was measured at 490 nm with the Thermo Varioskan Flash (Thermo Electron Corp., Vantaa, Finland). Cell viability was expressed as a percentage of control cells, which were defined as 100% viable.

CYP2E1 activity assay. Aniline hydroxylase (ANH) activity, a specific marker of CYP2E1 enzymatic activity, was spectrophotometrically measured with phenol as a coloring reagent (20). In brief, after treatment cells ($2x10^7$) were treated with RIPA buffer (Beyotime Institute of Biotechnology, Haimen, China), and protein concentrations were determined using the BCA protein assay kit (Beyotime Institute of Biotechnology). Then 10 mM aniline (Sigma) and 1 mM triphosphopyridine nucleotide (NADPH; Beyotime Institute of Biotechnology) were added to equal volumes of supernatants. After incubated for 1 h at 37˚C, equal volumes of 20% trichloroacetic acid (TCA; Sigma) were added to the mixture and maintained on ice for 5 min. Samples were centrifuged at 1902 x g for 10 min, and the supernatants were added to equal volumes of 5% phenol (Sigma) and 1% sodium carbonate (Sigma). The mixture was incubated for 60 min at RT. ANH activation resulted in producing 4-amino phenol, which was detected spectrophotometrically (630 nm) with the Thermo Varioskan Flash (Thermo Electron Corp.).

Quantitative RT-PCR. By resuspension in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) total RNA was extracted from cells. Relative quantification of the genes of interest was measured by real-time PCR using the Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific Inc., Waltham, MA, USA). Real-time PCR of the housekeeping gene β-actin allowed normalization of the expression of the genes of interest and expression relative to non-treated control samples was calculated utilizing the ΔΔCT method: Relative expression level=$2^{-\Delta\Delta CT}$, where ΔCT=Ct (gene of interest)-Ct (housekeeping gene), and ΔΔCT=ΔCT (experimental group)-ΔCT (control group). Reactions were carried out in a Stratagene Mx3000P qPCR System (Agilent Technologies Inc., Waldbronn, Germany). The CYP2E1 primers used for qPCR were: forward, 5'-GCCGAATCCCTGCCATCAA-3' and reverse, 5'-GGTGTCTCGGGTTGCTTCAT-3'. The β-actin primers used for qPCR were: forward, 5'-CGTGCGTGA CATTAAGGAGAA-3' and reverse, 5'-AGGAAGGAAGG CTGGAAGAG-3'.

Western blotting. Cells were lysed by RIPA buffer and the total proteins were quantified with a BCA protein assay kit (Beyotime Institute of Biotechnology). After SDS-PAGE electrophoresis proteins were transferred to a PVDF membrane (Millipore Corp., Billerica, MA, USA), which was blocked in blocking buffer and incubated with primary antibodies. The primary antibodies applied included rabbit monoclonal anti-human CYP2E1 (1:500 dilution, catalog no. ab151544, Abcam Inc., Cambridge, MA, USA) and mouse monoclonal anti-human β-actin (1:5000 dilution, catalog no. 3700, Cell Signaling Technology, Boston, MA, USA). The second antibody was the fluorescent secondary antibodies (Alexa Fluor® 790 goat anti-rabbit IgG, 1:10,000 dilution, Catalog no. 111-655-144; Alexa Fluor 680 goat anti-mouse IgG, 1:10,000 dilution, catalog no. 115-625-146; LI-COR Biosciences, Lincoln, NE, USA). The membranes were detected and analyzed with an Odyssey® CLx Infrared Imaging System (LI-COR Biosciences), and the results were analyzed with ImageJ software.

Immunohistofluorescence assay. Smmc-7721 cells, QGY-7703 cells and HepG2 cells were fixed with 2% paraformaldehyde (V:V; Sigma) at 37˚C for 30 min, and blocked with PBS^+ solution (PBS supplemented with 1% BSA) for 30 min at RT. Cells were then incubated at 37˚C for 1 h with rabbit monoclonal anti-human CYP2E1 (1:200 dilution, catalog no. ab151544, Abcam Inc.). Goat anti-rabbit IgG-FITC was used as secondary antibodies (1:100 dilution, catalog no. sc-2012, Santa Cruz Biotechnology Inc., Delaware, CA, USA), and nuclei were stained with Hoechst 33342 (Invitrogen, Eugene, OR, USA). Photomicrographs were captured with a Leica DMI 4000B microscope imaging system (Leica Microsystems, Wetzlar, Germany).

Inhibition of CYP2E1 activity. For inhibiting CYP2E1 activity, cells were pretreated with a CYP2E1 specific inhibitor, diethyldithiocarbamate (DDC; Sigma; 0.1 mM, final concentration) (21), at 3 h before indicated treatment.

siRNA transfection. Smmc-7721 cells were transfected with 100 nM of siRNA specific for human CYP2E1 siRNA (sc-270348, Santa Cruz Biotechnology Inc.) or control siRNA (sc-37007, Santa Cruz Biotechnology Inc.) using the Lipofectamine® 2000 Transfection Reagent (Thermo Fisher Scientific Inc., Rockford, IL, USA) as per the manufacturer's instructions. At 5 h after transfection, transfection medium was replaced with fresh medium containing serum and allowed to grow for 24 h. Whole cell extract was prepared 24 h after transfection for analysis of silencing efficiency by western blot using antibody against CYP2E1 (rabbit monoclonal anti-human CYP2E1, 1:500 dilution, catalog no. ab151544, Abcam Inc.).

Data analysis. All experiments were carried out at least three times. Data were expressed as the mean ± SD. The results were analyzed for statistical significance using ANOVA followed by Duncan's multiple-range test. Values of $p<0.05$ were considered to be statistically significant.

Results

EtOH (V:V) (4%) activates CYP2E1 in Smmc-7721 cells. Previous findings indicated that CYP2E1 could be activated by ethanol (EtOH) (22). As shown in Fig. 1A and B, no significant

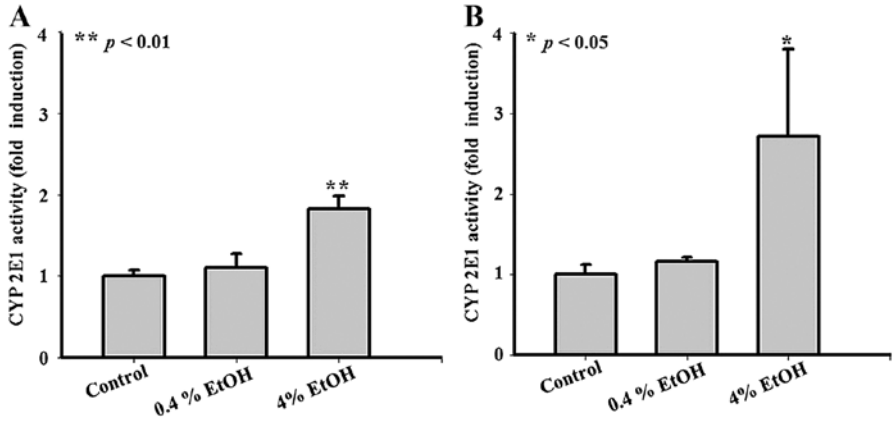

Figure 1. Effects of ethanol (EtOH) on the activity of cytochrome P450 2E1 (CYP2E1) in Smmc-7721 cells. Smmc-7721 cells ($2x10^7$) were cultured without or with 0.4% EtOH (V:V), or 4% EtOH (V:V) for 24 h (A) and 48 h (B). CYP2E1 activities were determined. Values are expressed as the mean ± SD of three determinations. *p<0.05, **p<0.01 versus the control without any treatment.

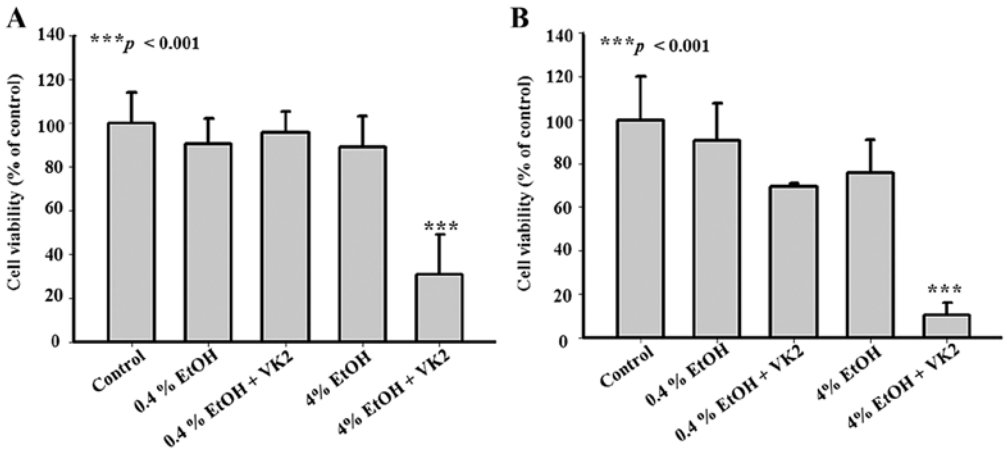

Figure 2. Effects of the combined treatment of Vitamin K2 (VK2) and EtOH on cell viability in Smmc-7721 cells. Smmc-7721 cells ($5x10^3$) were cultured without or with 0.4% EtOH, 4% EtOH, 0.4% EtOH plus 40 μM VK2, or 4% EtOH plus 40 μM VK2 for 24 h (A) and 48 h (B). Cell viabilities were measured by MTT assay. Values are expressed as the mean ± SD of three determinations. ***p<0.001 versus the control without any treatment.

changes of CYP2E1 activity were observed in Smmc-7721 cells treated with 0.4% EtOH (V:V) for 24 or 48 h compared with the untreated group. However, CYP2E1 activity increased to approximately 1.8-fold in cells treated with 4% EtOH for 24 h (Fig. 1A) and treated with 4% EtOH for 48 h CYP2E1 activity increased to approximately 2.7-fold (Fig. 1B).

EtOH (4%) enhances the inhibitory effect of VK2 on cell viability in Smmc-7721 cells. We examined the effects of the combined treatment of VK2 and EtOH on the viability of Smmc-7721 cells. As shown in Fig. 2A, compared with the untreated group, no significant changes of cell viability were observed in cells treated with 0.4% EtOH, 4% EtOH, or 40 μM VK2 plus 0.4% EtOH for 24 h. However, cell viability decreased to around 30% in cells treated with 40 μM VK2 plus 4% EtOH for 24 h (p<0.001) (Fig. 2A). The same trend was also observed in cells treated for 48 h (Fig. 2B). These results showed that 4% EtOH could enhance inhibitory effect of VK2 on the cell viability in Smmc-7721 cells.

CYP2E1 inhibition attenuates the synergistic effect of VK2 and EtOH in Smmc-7721 cells. CYP2E1 specific inhibitor and CYP2E1 siRNA were used to examine the effect of CYP2E1 on the synergistic effect of VK2 and EtOH. As shown in Fig. 3A and B, 0.1 mM DDC inhibited CYP2E1 activity and attenuated the synergistic effect of VK2 and EtOH. Moreover, the same attenuated effect was observed after CYP2E1 silencing by CYP2E1 siRNA (Fig. 3C and D).

Synergistic effect of VK2 and EtOH was also observed in QGY-7703 cells, but not in HepG2 cells, which did not express CYP2E1. In order to identify whether the synergistic effect of VK2 and EtOH exists in other hepatoma cells, we exposed QGY-7703 cells and HepG2 cells to VK2 plus EtOH. As shown in Fig. 4A, 4% EtOH enhanced the inhibitory effect of VK2 on the cell viability in QGY-7703 cells treated for 24 h. However, in HepG2 cells treated for 24 h, 4% EtOH had no significant effect on the inhibitory effect of VK2 (Fig. 4B). Previous findings reported that HepG2 cells did not express CYP2E1 (23,24). Furthermore, exposure of HepG2 cells to EtOH has been shown to have little effect on the induction of CYP2E1 (24,25). As shown in Fig. 4C and D, we also showed that the protein level of CYP2E1 in HepG2 cells was lower than that in Smmc-7721 cells or QGY-7703 cells.

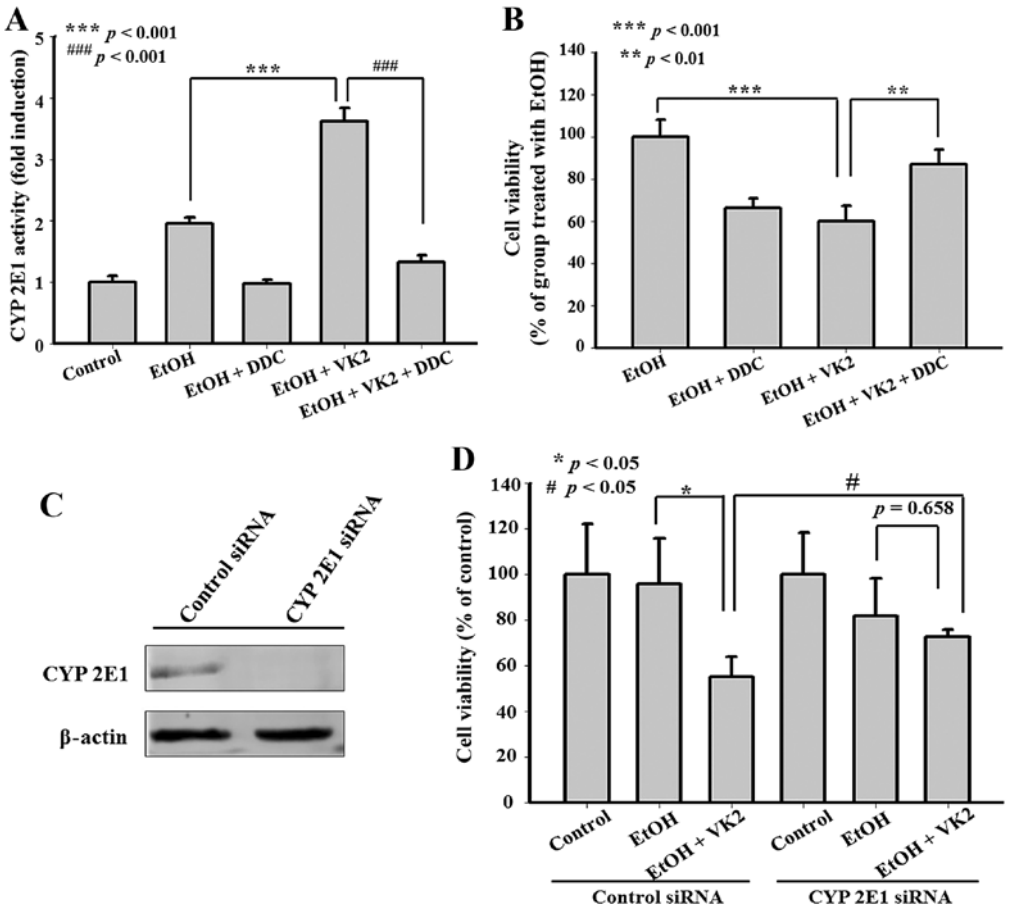

Figure 3. CYP2E1 inhibition attenuated the synergistic effect of VK2 and EtOH in Smmc-7721 cells. (A) Diethyldithiocarbamate (DDC), a specific CYP2E1 inhibitor, inhibited the activity of CYP2E1. Values are expressed as the mean ± SD of three determinations. ***p<0.001 versus cells treated with 4% EtOH. $^{###}$p<0.001 versus cells treated with 4% EtOH plus 40 μM VK2. (B) DDC attenuated the inhibitory effect of VK2 on cell viabilities. Values are expressed as the mean ± SD of three determinations. ***p<0.001 versus cells treated with 4% EtOH. **p<0.01 versus cells treated with 4% EtOH plus 40 μM VK2. (C) CYP2E1 was silenced by CYP2E1 siRNA. (D) Silencing of CYP2E1 attenuated the inhibitory effect of VK2 on cell viabilities. Values are expressed as the mean ± SD of three determinations. *p<0.05 versus cells treated with 4% EtOH. $^{\#}$p<0.05 versus cells treated with control siRNA.

VK2 increases CYP2E1 activity and results in upregulation of CYP2E1 expression. In order to identify the effect of VK2 on CYP2E1, CYP2E1 activity and expression were examined in Smmc-7721 cells treated with different concentrations of VK2. As shown in Fig. 5A and B, 40 μM VK2 increased CYP2E1 activity in cells treated for 24 h and 48 h. Although no significant changes of CYP2E1 expression were observed in cells treated for 24 h, 40 μM VK2 significantly upregulated CYP2E1 expression in cells treated for 48 h (Fig. 5C and D).

Discussion

VK2 is a natural and main form of vitamin K in the tissue. It has been approved as an anti-osteoporotic medicine by the Ministry of Health, Labor and Welfare in Japan. Moreover, the safety of the long-term administration of VK2 has been well established (26). Although the exact mechanism has not yet been elucidated in detail, VK2 and their analogs have been shown to inhibit the survival of various cancer cell lines (27-29). However, a previous study reported that hepatoma cells were insensitive to VK2 and, even at higher concentrations (>100 μM) VK2 could not exhibit significant inhibitory effect or induce apoptosis in hepatoma cells (14). As VK2 is a natural, safe and clinically-utilized agent, we searched for substances that could enhance the inhibitory effect of VK2 in hepatoma cells. We found that EtOH, which resulted in an increase of CYP2E1 activity, could enhance the inhibitory effect of VK2 on the cell viability in Smmc-7721 cells. CYP2E1 is one of the important hepatic metabolic enzymes, which is responsible for the catalysis of xenobiotic (30). Previous studies showed that CYP2E1 could synergize and increase the susceptibility of hepatic cells to different chemicals (18,19). Overexpression of CYP2E1 could enhance sensitivity of hepG2 cells to fas-mediated cytotoxicity (31). In the present study, we also showed that CYP2E1 inhibition attenuated the synergetic effect of VK2 and EtOH in Smmc-7721 cells. These findings indicate that CYP2E1 may be an attractive target for enhanced sensitivity of hepatoma cells to VK2.

At present, the effects of CYP2E1 on hepatoma are limited. Previous studies showed that the expression of CYP2E1 in tumor cells tended to decrease with the decrease of cell differentiation degree, and was the lowest in poorly differentiated HCC (32,33). Ho *et al* reported that decreased expression of CYP2E1 was associated with poor prognosis of hepatocellular carcinoma (34). In several HCC cell lines the expression of CYP2E1 was absent or weak (34,35). Furthermore, previous studies showed that overexpression of CYP2E1 induced or enhanced cytotoxicity to HepG2 cells,

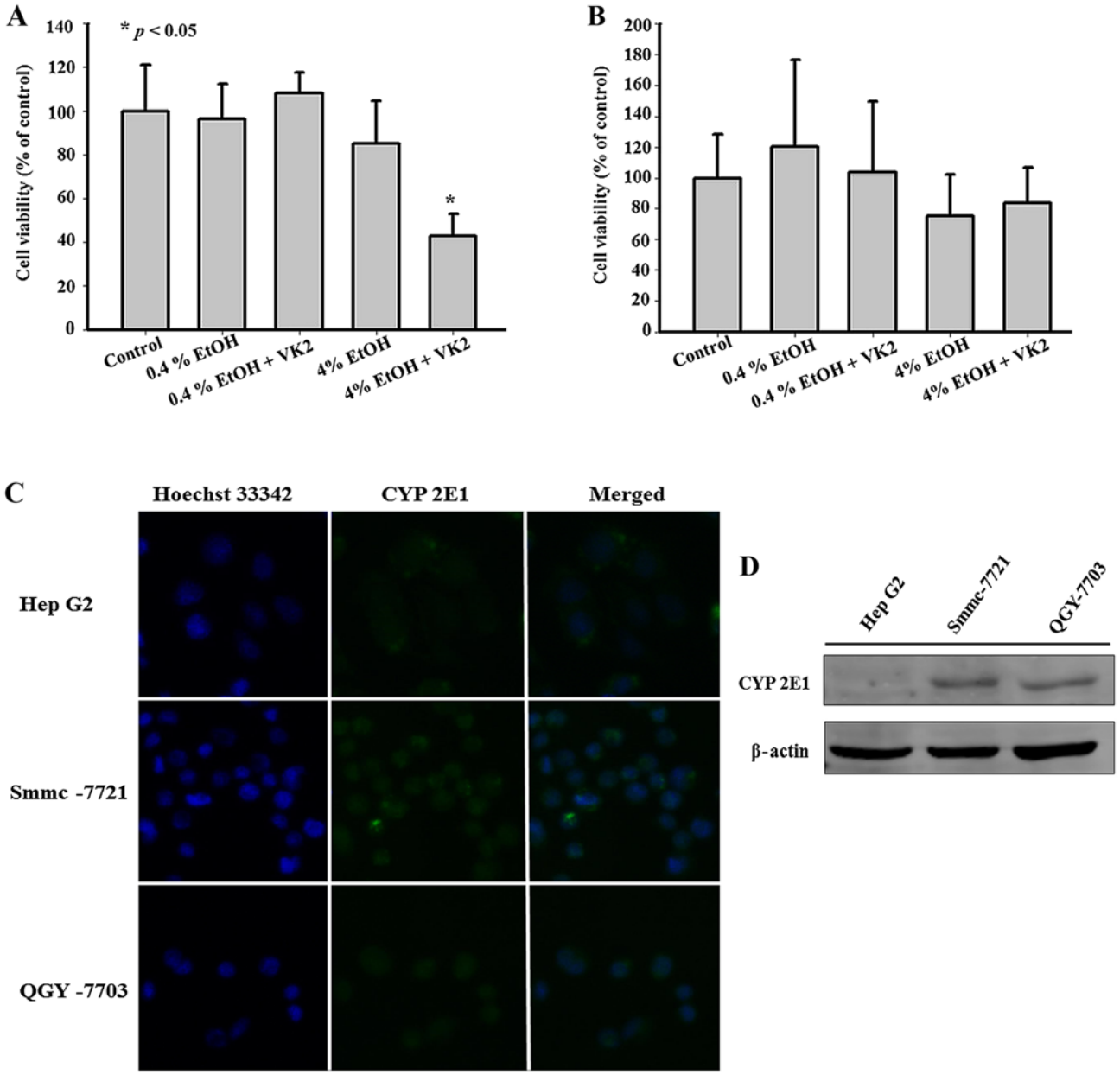

Figure 4. Effects of the combined treatment of VK2 and EtOH on cell viability in other hepatoma cells. (A) QGY-7703 cells ($5x10^3$) and (B) HepG2 cells ($5x10^3$) were cultured without or with 0.4% EtOH, 4% EtOH, 0.4% EtOH plus 40 μM VK2, or 4% EtOH plus 40 μM VK2 for 24 h. Cell viabilities were measured by MTT assay. Values are expressed as the mean ± SD of three determinations. (C and D) The protein levels of CYP2E1 were examined by immunohistofluorescence assay and western blotting in three hepatoma cell lines.

in which the level of CYP2E1 is originally very low (31,36). In the present study, we also showed that in those cells with higher levels of CYP2E1, such as Smmc-7721 and QGY-7703 cells, the antitumor effect of VK2 was significantly enhanced by EtOH. However, in cells with lower levels of CYP2E1, such as HepG2 cells, EtOH had no influence on antitumor effects of VK2. These results suggested that induction of CYP2E1 might favor HCC treatment.

However, previous findings reported that induction of CYP2E1 caused oxidative stress and resulted in hepatic cytotoxicity induced by alcohol or other hepatotoxicants (37-39). Robertson *et al* reported that in nonalcoholic steatohepatitis CYP2E1 affected the cell viability of hepatocytes (40). It is undesirable that induction of CYP2E1 results in cytotoxicity to hepatocytes. The effect of CYP2E1 on the hepatic cells is fairly complex. Schattenberg *et al* reported that CYP2E1 served both to protect against and to promote cellular injury in hepatocytes (18). Overexpression of CYP2E1 sensitized hepatocytes to necrotic death from the polyunsaturated fatty acid, but surprisingly protected hepatocytes against vitamin K3 (VK3)-induced apoptotic death (18). Since VK2 and VK3 belong to vitamin K, this protection of hepatocytes from VK3-induced cell death inspires that induction of CYP2E1 may protect hepatocytes from VK2-induced cell death. In fact, our further study showed that VK2 plus 4% EtOH could induce apoptosis in hepatoma cells, but had no significant effect on hepatocytes (data not shown).

CYP2E1 can be regulated at different levels via various mechanisms. Many studies reported that CYP2E1 protein activity were often induced by its own substrates through

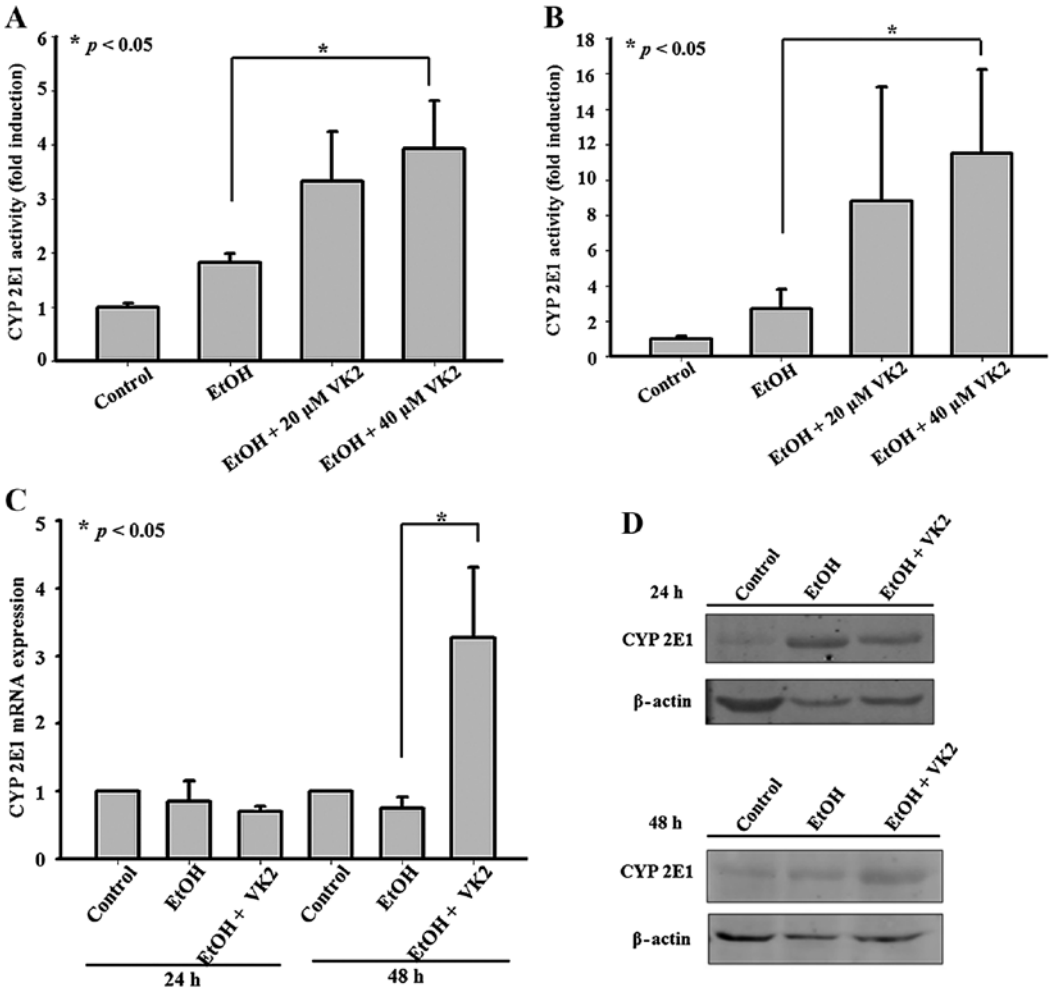

Figure 5. Effects of VK2 on CYP2E1. Treated for 24 h (A) and 48 h (B) VK2 increased CYP2E1 activity in Smmc-7721 cells. Values are expressed as the mean ± SD of three determinations. *p<0.05 versus cells treated with 4% EtOH. (C) CYP2E1 gene expression was examined by RT-qPCR in cells treated for 24 and 48 h. Values are expressed as the mean ± SD of three determinations. *p<0.05 versus cells treated with 4% EtOH. (D) The protein levels of CYP2E1 were examined by western blotting in cells treated for 24 and 48 h.

post-transcriptional mechanisms (41). Post-transcriptional regulation also involved CYP2E1 mRNA stabilization (42). Some studies showed that CYP2E1 transcription was influenced by a variety of compounds such as IL-6, T3 and insulin (42-44). In the present study, our results showed that VK2-induced initial increase of CYP2E1 activity is not due to *de novo* synthesis, suggesting that CYP2E1 could be induced by VK2 via post-transcriptional regulation. Herein, we also showed that CYP2E1 could be transcriptional regulated by VK2, but the molecular mechanism is still poorly understood.

In conclusion, our results suggest that CYP2E1 induction can enhance the inhibitory effect of VK2 on hepatoma cells. CYP2E1 may be an attractive target for enhanced antitumor effects of VK2 in HCC. It enlightens us that percutaneous injection of VK2 plus CYP2E1-inducers by ultrasonic guidance may be an economic effective treatment for HCC patients without prominent adverse effects.

Acknowledgements

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