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ORIGINAL RESEARCH

Anti-proliferative and pro-apoptotic effect of carvacrol on human hepatocellular carcinoma cell line HepG-2

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Abstract Carvacrol is one of the members of monoterpene phenol and is present in the volatile oils of *Thymus vulgaris*, *Carum copticum*, origanum and oregano. It is a safe food additive commonly used in our daily life, and few studies have indicated that carvacrol has anti-hepatocarcinogenic activities. The rationale of the study was to examine whether carvacrol affects apoptosis of human hepatoma HepG2 cells. In this study, we showed that carvacrol inhibited HepG2 cell growth by inducing apoptosis as evidenced by Hoechst 33258 stain and Flow cytometric (FCM) analysis. Incubation of HepG2 cells with carvacrol for 24 h induced apoptosis by the activation of caspase-3, cleavage of PARP and decreased Bcl-2 gene expression. These results demonstrated that a significant fraction of carvacrol treated cells died by an apoptotic pathway in HepG2 cells. Moreover, carvacrol selectively altered the phosphorylation state of members of the MAPK superfamily, decreasing phosphorylation of ERK1/2 significantly in a dose-dependent manner, and activated phosphorylation of p38 but not affecting JNK MAPK phosphorylation. These results suggest that carvacrol may induce apoptosis by direct activation of the mitochondrial pathway, and the mitogen-activated protein kinase pathway may play an important role in the antitumor effect of carvacrol. These results have identified, for the first time, the biological activity of carvacrol in HepG2 cells and should lead to further development of carvacrol for liver disease therapy.

Keywords Carvacrol · Hepatocellular carcinoma · Apoptosis · Mitochondrial pathway · Mitogen-activated protein kinase pathway

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Introduction

Liver cancer is the sixth most common cancer in the world and the third most common cause of malignancy with 598,000 deaths every year (Parkin et al. 2005). Hepatocellular carcinoma (HCC), which is the predominant type, is an increasingly common malignancy with a poor prognosis and limited systemic treatment options, approximately 80% of patients die within a year of diagnosis (Lammer et al. 2010). The emerging incidence rates of HCC are in sub-Saharan

Africa and Eastern Asia (>80% of all HCC), with China accounting for 55% of the cases (McGlynn and London 2005). Therefore, a more effective therapeutic strategy for human hepatocellular carcinoma is urgently needed.

A good chemopreventive agent is a naturally occurring agent that can induce apoptosis in cancer cell without much side effects (Surh 1999). In the past decades, a number of studies have successfully evaluated that several natural products activated cell death signals and induced apoptosis in cancer cells, including hepatocellular carcinoma. Carvacrol (2-methyl-5-(1-methylethyl)-phenol) (Fig. 1) is a natural member of monoterpene phenol and is present in the volatile oils of *Thymus vulgaris*, *Carum copticum*, origanum and oregano (Martins et al. 1999; Kisk and Roller 2005; Lampronti et al. 2006). It has been safely used in our daily life: such as cosmetic ingredient, safe food additive in baked goods, sweets, beverages, and chewing gum. As well known, carvacrol has been shown to exhibit anti-microbial, anti-mutagenic, anti-platelet, analgesic, anti-inflammatory, anti-angiogenic, anti-oxidant, anti-elastase, insecticidal, anti-parasitic,cell-protective, AChE inhibitor and anti-tumor activity (Aligiannis et al. 2001; Sokmen et al. 2004; Can Baser 2008). In the recent past, its antitumor effect has arisen the concern of investigator. It was revealed that carvacrol has anti-proliferative properties on non-small cell lung cancer cells, A549, chronic myeloid leukemia cells, K562, Hep-2 cells, murine B16 melanoma cells and human metastatic breast cancer cells, MDA-MB231 (He et al. 1997; Koparal and Zeytinoglu 2003; Lampronti et al. 2006; Karkabounas et al. 2006; Horvathova et al. 2007; Arunasree 2010).Based upon the previous studies, it has been shown that carvacrol had a broad-spectrum of cytotoxic and anticancer activity. However, the mechanism of its anti-cancer activity is not yet elucidated.

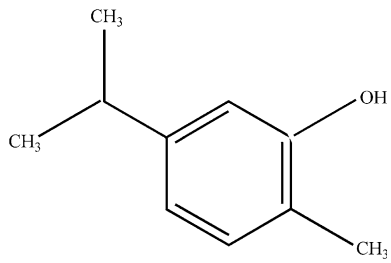

Fig. 1 The structure of carvacrol (2-methyl-5-(1-methylethyl)-phenol)

Thus the primary objective of our study was to examine a systematic, detailed study of the effects of carvacrol on human hepatocellular carcinoma cell line HepG-2.

Cancer is a very complex disease. It has been proven that the occurrence and development of tumor cells is closely related to abnormal intracellular signal transduction system (Wolf et al. 2007). Mitogen-activated protein kinase (MAPK) is one of the major signaling systems that transduce extracellular signals into cells (Binetruy et al. 2007). There are at least three major subfamilies of MAPK: the extracellular-signal-regulated kinases (ERK), the c-jun N-terminal kinase or stress-activated protein kinases (JNK or SAPK), and p38 MAPK. All of these activated by dual phosphorylation and play essential roles in the regulation of intracellular metabolism, gene expression and integral actions in many aspects including cell growth, differentiation, apoptosis and cellular responses to external stresses (Pearson et al. 2001). A great deal of evidence has indicated that over expression and activation of MAPKs are extremely important in the development of cancer (Huang et al. 2004), so the MAPK pathway function as a possible target for newly-designed anti-tumor drugs have attracted considerable attention in recent years. Therefore, in order to provide experimental data for further research on the signal transduction of apoptosis induced by carvacrol in human hepatocellular carcinoma cell, we examined the activity of ERK, p38, and JNK after carvacrol-induced differentiation in the human hepatocellular carcinoma cell line HepG-2.

Materials and methods

Materials and cell culture

Carvacrol (98%) was purchased from Sigma–Aldrich (CAS Number: 499-75-2), RPMI 1640 medium and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA), DMSO and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) from Amresco (Solon, OH, USA). Antibodies against caspase-3, PARP, Bcl-2, phosphop-38 mitogen-activated protein kinases (MAPK) (Thr180/Tyr182), phospho-JNK, phospho-ERK1/2 (p42/44 MAPK) (Thr202/Tyr204), ERK, p38, JNK were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). All other chemicals were

obtained from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise specified.

Cell line and culture

The human hepatocellular carcinoma cell line HepG-2 and hepatocyte LO2 cells (human fetal normal liver cells) were provided by the Cancer Research Institute of the University of South China (Hengyang China). Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). The cells were grown at 37 °C humidified incubator with an atmosphere of 5% CO_2.

MTT-cell proliferation assay

The effect of carvacrol on the viability of HepG-2 and LO2 cells was determined by 3-(4, 5-dimethylthiazol-2-yl) -2, 5 -diphenyltetrazoliumbromide (MTT) assay. The cells were seeded in 96-well culture plates at the density of 5×10^3 cells/well. After overnight incubation, carvacrol was added, and the final volume was 200 μl. Each concentration was repeated five times. After treatment, the medium was removed and 20 μl of MTT (5 mg/ml in PBS) was added to each well. After 4 h incubation at 37 °C, the medium was removed and formazan was dissolved in 150 μl of DMSO and then the plates were vigorously shaken to ensure complete solubilization. Formazan absorbance was assessed at 490 nm using a microplate reader (Elx-800, Bio-Tek). The survival rate of HepG-2 cells was calculated according to the equation as following: $A_{\text{Treated group}}/A_{\text{Control group}} \times 100\%$. All experiments were performed in triplicate.

Cell morphological assay

Apoptosis was measured by Hoechst 33258 staining using the kit from Beyotime Institute of Biotechnology (Jiangsu, China). In brief, after being treated with carvacrol, the cells were washed with PBS, 5 μl of 10 mg/ml Hoechst 33258 was added to the solution in each well and the plates were incubated for 10 min at 37 °C in the dark. The cells were then observed under a fluorescence microscope (Olympus, Tokyo, Japan) with a FITC filter set.

Flow cytometry (FCM) assay

Cells were treated with carvacrol 24 h, then collected, and washed twice with PBS, and then fixed with 70% ethanol (v/v) overnight at 4 °C. The cells were again rinsed with PBS and incubated with 50 μg/ml RNase A and 50 μg/ml propidium iodide (PI) solution (Sigma, American). The samples were kept in the dark at 4 °C for 30 min and then analyzed using a flow cytometer (Coulter epics altra HyperSort™ system, USA). The resulting DNA histograms were quantified using Cell Quest Pro software.

Western blot analysis

Cells were collected and then washed twice with cold PBS and lysed with buffer (5×10^4 μmol L^{-1}, Tris–HCl and 5×10^4 μmol L^{-1} NaCl, pH 8.0) and sonicated for 15 min on ice. After measuring the protein concentrations, equal amounts of protein from extracts were resolved by 10–15% sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS–PAGE) and transferred to Immobilon-P PVDF membranes (Millipore Corporation, Bedford, MA, USA). Then the membranes were blocked with 5% skimmed milk for 4 h at room temperature with constant shaking, the membranes were incubated overnight at 4 °C with anti-caspase-3 antibody, anti-PARP antibody, anti-Bcl-2 antibody, anti-phospho-p38 MAPK antibody (Thr180/Tyr182), anti-phospho-JNK antibody, anti-phospho-ERK1/2 antibody (Thr202/Tyr204), anti-ERK antibody, anti-p38 antibody, and anti-JNK antibody. The membranes were washed three times with TBST, then probed with horseradish peroxidase-conjugated secondary antibody. The membrane was incubated for 1 minute in enzyme chemiluminescence (ECL) substrate and the exposed to X-ray film at for initial 10-second exposure time. Simultaneously, the FluorChem M (Cell Biosciences Supplies) system was used.

Inhibitor treatment

To detect the effect of MAPK on carvacrol-induced apoptosis and anti-proliferative activity in HepG2 cells, 10 μmol L^{-1} U0126 (an ERK-specific inhibitor) and 10 μmol L^{-1} SB203580 (a p38-specific inhibitor) was preincubated for 2 h before the addition of carvacrol by MTT analysis and morphological observation.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test when the F value was significant. The results were expressed as the mean ± SD. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software (Release 13.0, SPSS Inc.). Differences were considered statistically significant if $p < 0.05$.

Results

Effect of carvacrol on viability of HepG2 cells

The inhibitory effect of carvacrol was estimated by measurement of the viability of HepG2 cells and LO2 cells. The treatment of LO2 cells with carvacrol (0–0.4 mmol L^{-1}) for 24 h was without significant effect on cell viability. However, treatment of HepG2 cells with similar doses of carvacrol, the viability of HepG-2 cells was decreased after treatment with 0.05, 0.1, 0.2, or 0.4 mmol L^{-1} carvacrol. As shown in Fig. 2, Carvacrol inhibited cell proliferation in HepG2 cell lines in a concentration-dependent manner. The IC_{50} for carvacrol was approximately estimated to be 0.4 mmol L^{-1} for HepG-2 cells. Therefore, the results demonstrated that carvacrol could cause obvious growth inhibition of HepG2 cells at the tested range of concentrations and showed no cytotoxic to the LO2 cells. Previous experiments also proved that carvacrol had no significant effects on normal lung cells (HFL1 cells), but the same procedure was applied to non-small cell lung cancer cells (A549) leading to inhibition of proliferation and induction of early apoptotic features in a dose-dependent manner (Koparal and Zeytinoglu 2003).

Effect of carvacrol on apoptosis in HepG2 cells

To determine whether carvacrol induces apoptosis, morphological observation and flow cytometer were performed. As shown in Fig. 3a, uniform HepG-2 cells with normal morphology were observed in the control group, whereas HepG-2 cells with fragmented chromatin and apoptotic bodies were observed followed by the treatment with 0.05, 0.1, 0.2 mmol L^{-1} or 0.4 mmol L^{-1} carvacrol (Fig. 3a). Results from previous studies have shown similar effects of carvacrol

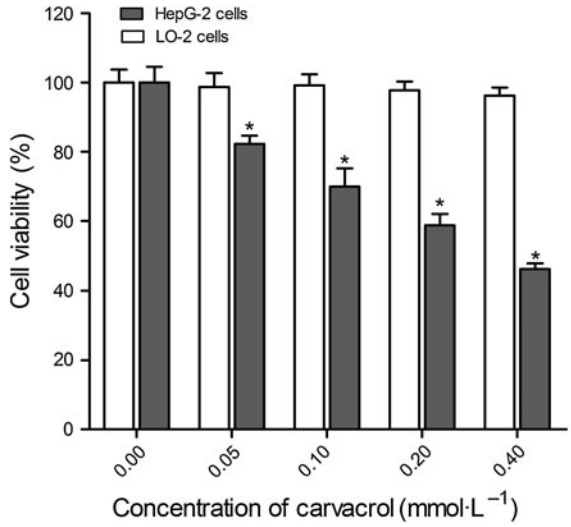

Fig. 2 Cell survival was examined by MTT assay. HepG-2 and LO2 cells were treated with different concentrations of carvacrol (0.05, 0.1, 0.2, 0.4 mmol L^{-1}) for 24 h. Cell growth was determined by MTT assay and was directly proportional to the absorbance at a wave length of 490 nm. Values are expressed relative to the control and are means ± SD, n = 3. Means without a common letter differ, $^*P < 0.05$

on non-small cell lung cancer cells (A549) and metastatic breast cancer cells (MDA-MB231) (Arunasree 2010). Apoptotic cells were measured with PI staining of DNA fragmentation by flow cytometry (Yuan et al. 2004). As shown in Fig. 3b, the apoptosis rate increased from 13.5 to 25.8%, 30.5 and 50.6% respectively by the treatment of carvacrol for 24 h. Taken together, these results suggest that carvacrol is capable of inducing marked apoptosis in HepG-2 cells.

Effect of carvacrol on the expression of apoptosis-related proteins

Among the proteins that play a key role in the regulation of apoptosis and that are affected by various apoptosis inducing agents are the members of the Bcl-2 superfamily. In order to see whether carvacrol affected the expression of the anti-apoptotic Bcl-2 protein, total proteins were extracted from untreated and carvacrol-treated HepG2 cells and analyzed for the expression of Bcl-2. Western blot analysis showed that carvacrol down regulated the expression of Bcl-2 protein levels which were reduced with increasing concentrations of carvacrol (Fig. 4a). Other hallmarks of cells undergoing

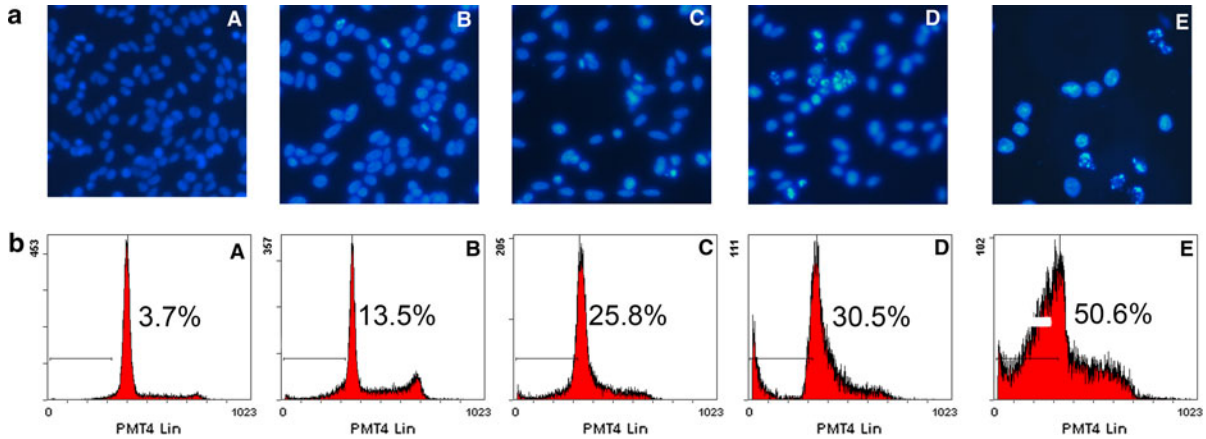

Fig. 3 Effect of carvacrol on apoptosis in HepG2 cells. **a** Morphological effects on HepG2 cells stained with Hoechst 33258 fluorescence assay. (× 400): (*A*) Untreated HepG-2 cells. (*B*) Cells treated with 0.05 mmol L^{-1} carvacrol for 24 h. (*C*) Cells treated with 0.1 mmol L^{-1}carvacrol for 24 h. (*D*) Cells treated with 0.2 mmol L^{-1} carvacrol for 24 h. (*E*) Cells treated with 0.4 mmol L^{-1} carvacrol for 24 h **b** Cells were collected and stained with propidium iodide (PI) and analyzed by flow cytometry. HepG-2 cells were treated for 24 h with (*A*) control, or the following concentrations of carvacrol: (*B*) 0.05 mmol L^{-1}; (*C*) 0.1 mmol L^{-1}; (*D*) 0.2 mmol L^{-1}; (*E*) 0.4 mmol L^{-1}

apoptosis by a variety of apoptotic stimuli are activation of caspase-3 and cleavage of poly-(AD-Pribose)-polymerase (PARP). Western immunoblot analysis showed that a significant activation of caspase-3 occurred at 0.2 mmol L^{-1} carvacrol after 24 h of incubation, and the Caspase-mediated PARP-cleavage by carvacrol-induced apoptosis also was a concentration-dependent process (Fig. 4a). Collectively, the results obtained from proliferation (Fig. 2) and apoptosis (Figs. 3 and 4a) assays suggested that carvacrol effectively inhibited cell proliferation and induced apoptosis by mitochondria-mediated apoptosis pathway.

Effect of carvacrol on the activation of MAPKs

To evaluate the possible roles of MAPKs in carvacrol induced cell apoptosis of HepG2 cells, levels of phosphorylated MAPKs were investigated. Human hepatocellular carcinoma cell HepG-2 were treated with 0.05, 0.1, 0.2, 0.4 mmol L^{-1} carvacrol for 15 min. The cells were harvested and lysed, and the phosphorylation levels of MAPKs, JNK, p38 MAPK, and ERK1/2 were analyzed by western blot. As shown in Fig. 4b, the phosphorylation of ERK1/2 decreased significantly in a concentration-dependent manner. However, the phosphorylation level of p38 MAPK increased and JNK did not change under the same conditions. Accordingly, ERK and p38

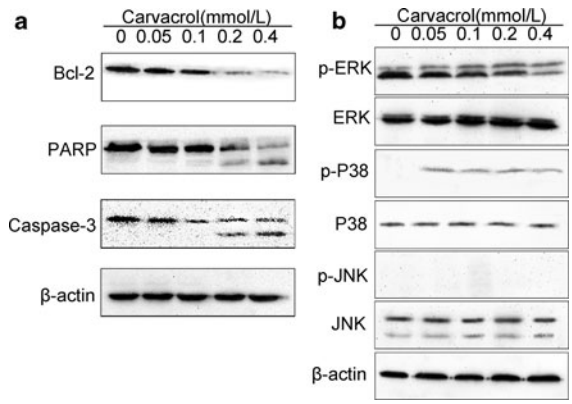

Fig. 4 Effects of carvacrol on apoptosis-related proteins and MAPK protein levels in HepG-2 cell. **a** Western blot analysis of the expression of Bcl-2, activation of caspase-3 and cleavage of PARP. **b** Carvcrol-induced phosphorylation of MAPK in HepG2. Carvacrol downregulated the expressions of p-ERK, and upregulated the expressions of p-P38 but no obvious effect on the expressions of p-JNK

signaling pathways might be involved in the response of HepG2 cells to carvacrol.

Effects of Inhibitors on carvacrol-induced apoptosis

To further analyse the effect the possible roles and effects of MAPKs in carvacrol-induced apoptosis, we examined changes in the cell viability and morphological changes

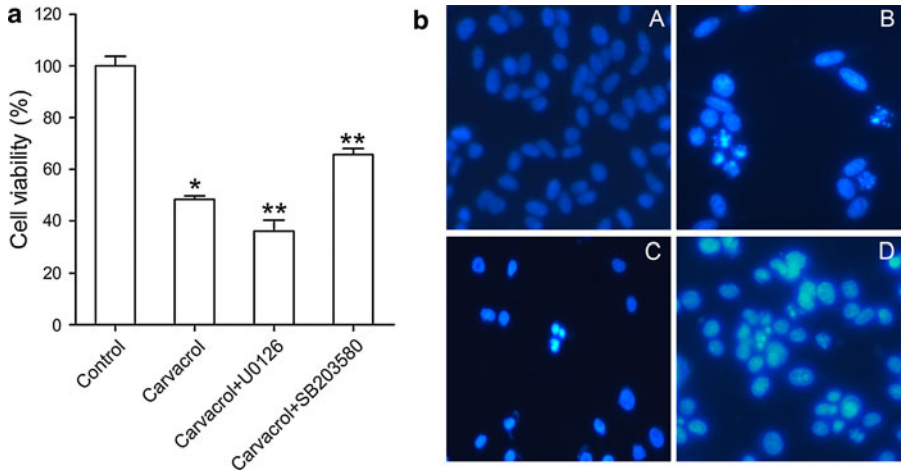

Fig. 5 Effects of carvacrol and MAPK inhibitors on carvacrol-induced anti-proliferation activity and apoptosis in HepG2 cells. **a** Carvacrol-induced proliferative suppression in the presence of MAPK inhibitors was determined by MTT assay. $*P < 0.05$, compared with control group, $**P < 0.05$ compared with carvacrol-treated group. **b** Roles of MAPK inhibitors in carvacrol-induced apoptosis. (*A*) Control (*B*) 0.4 mmol L^{-1}carvacrol; (*C*) 0.4 mmol L^{-1}carvacrol + 10 μmol L^{-1}U0216; (*D*) 0.4 mmol L^{-1} + 10 μmol L-1 SB203580

due to apoptosis in the presence or absence of specific inhibitors for ERK (U0126) and p38 MAPK (SB203580). From the MTT analysis, SB203580 prominently reversed carvacrol (0.4 mmol L^{-1})-induced cell death (Fig. 5a), which was concomitant with a decrease in the appearance of apoptotic bodies induced by carvacrol (Fig. 5b). Pretreatment with U0126 significantly promoted carvacrol-induced anti-proliferative activity in HepG2 cells (Fig. 5a), but no increase in the appearance of apoptotic bodies induced by carvacol was observed. These results suggested that the ERK pathway may be involved in the anti-proliferative mechanism, while the p38 pathway might participate in the induction of apoptosis caused by carvacrol in HepG2 cells.

Discussion and conclusion

Nowadays, one of the main methods of modern cancer treatment is chemotherapy (sometimes cancer chemotherapy). Most chemotherapeutic agents for cancer have different substantial short and long term side effects. Thus, in recent years major research has been focused on components isolated from herbs and plants which have been considered for being nontoxic and for the prevention and treatment of certain types of cancer (Kinghorn et al. 2004; Sarkar and Li 2004; Douer and Tallman 2005).

Previous studies have shown that carvacrol can exhibit strong antitumor activity. They revealed that carvacrol suppresses the growth of mouse B16 melanomas (He et al. 1997) and human larynx carcinoma Hep-2 cells (Stammati et al. 1999) in vitro and was active against DMBA-induced lung tumors in rats at 0.1 mg/kg i.p. dose (Zeytinoglu et al. 1998). Additional evidence for its antitumor activity was found: inhibition of DNA synthesis in mouse myoblasts bearing a human N-ras oncogene (Zeytinoglu et al. 2003). The further study indicated that carvacrol, dose-dependently (100, 250, 500 and 1,000 μmol L^{-1}), decreased the number of cancer cells, and total protein content and increased the degeneration of cell morphology of the human non-small cell lung cancer (NSCLC) cell line A549 (Koparal and Zeytinoglu 2003). Correspondingly, the same treatment was applied to HFL1 cells showing non-significant effects on these cells.. Subsequently, many experiments also confirmed similar effects of carvacrol on leiomyosarcoma cells (Karkabounas et al. 2006) and chronic myeloid leukemia cells, K562 (Horvathova et al. 2007). Although the anti-tumor activities of carvacrol have been demonstrated earlier, the mechanisms behind this activity are still not very clear. Some findings suggested that the antitumor activity of carvacrol was not due to cytotoxicity but possibly due to prevention of prenylation of several proteins including ras (Zeytinoglu et al. 2003), some results showed that its

mechanism may be due to its antioxidant nature (Ipek et al. 2003). Recently, the latest research showed that the anti-proliferative effects of carvacrol in metastatic breast cancer cells (MDA-MB231) was based on the activation of the classical apoptosis response, including decrease in mitochondrial membrane potential and increase in cytochrome c release from mitochondria, decrease in Bcl-2/Bax ratio, increase in caspase activity and cleavage of PARP and fragmentation of DNA, which belong to the mitochondrial pathway of the apoptosis pathway (Arunasree 2010). However, the effect of carvacrol on hepatocellular carcinoma was still unknown. In our study, we found that carvacrol induced stronger effects on hepatocellular carcinoma cells compared to normal human fetal liver cells. It did not show cytotoxicity to normal cells. Similarly, previous studies have proven that carvacrol show different cytotoxicity on lung cancer and normal lung cells (Koparal and Zeytinoglu 2003). Then, in order to well characterize and to explore the mechanisms responsible for the effects of carvacrol, HepG2 cells were incubated with different concentrations of carvacrol for 24 hours, and the morphology of HepG2 cells was followed by nuclear staining with Hoechst 33258. The result showed that there is an increase in the number of apoptotic cells after carvacrol treatment as suggested by cell shrinkage, chromatin condensation, nuclear fragmentation and shedding. A flow cytometry DNA analysis revealed that the apoptosis induced by carvacrol increased notably. Apoptosis is accompanied by caspase-3 mediated cleavage of PARP to generating an 85 kDa fragment (Enari et al. 1998). Cleavage of PARP is considered to be involved at least indirectly in triggering apoptosis. Furthermore, it has been demonstrated that members of the Bcl-2 family of proteins are critical regulators of the apoptotic pathway. Previous studies have shown that after treatment of MDA-MB231 cells with carvacrol Bcl2/Bax ratio decreased, caspase activity increased, and cleavage of PARP and fragmentation of DNA increased. Our experiments demonstrated similar changes in carvacrol treated HepG-2 cells. To further understand the possible mechanisms for the effects of carvacrol, we focused on the MAPK pathway as the possible mechanism for apoptosis induction. MAPKs are the major molecular players in cell cycle progression. There is now substantial evidence showing that the MAPK pathway signaling is important in experimental HCC. The MAPK pathway, especially JNK, ERK, and p38, has various functions in the apoptosis of various cancer cells (Xia et al. 1995; Hagemann and Blank 2001; Simstein et al. 2003; Yoon and Seger 2006). It is universally accepted that activation of ERK enhances cell proliferation (Junttila et al. 2008), while activation of JNK and/or p38 MAPK induces cell death and apoptosis (Lee et al. 2005; Gopalakrishnan et al. 2006; Chang et al. 2008). Previous research has shown that carvacrol effects involve ERK and the phosphorylation of p38 MAPK and JNK (Chan et al. 2005; Lee et al. 2008). Our results showed that carvacrol could decrease the phosphorylation of ERK1/2 MAPK and activate phosphorylation of p38 MAPK but showed no effect on JNK MAPK in HepG2 cells, and their role in carvacrol-induced apoptosis is still unclear. Therefore, we used p38 MAPK and ERK respective inhibitors to analyze the roles of MAPK participation in carvacrol-induced apoptosis. We found that after blocking the activation of p38 carvacrol-induced anti-proliferation of HepG2 cells was increased, and the number of apoptotic cells was reduced after pre-incubation with the p38 kinase-specific inhibitor, SB203580. However, pretreatment with the ERK kinase-specific inhibitor, U0216, resulted in an enhancement of the growth inhibition of HepG2 cells, moreover, and no more carvacrol-induced apoptosis in HepG-2 cells was observed. Thus, we can infer that the ERK pathway has an anti-proliferative action, and the p38 pathway is involved in the induction of apoptosis.

In summary, this is the first evidence for the effect of carvacrol on apoptosis in hepatocellular carcinoma cells, and the mitogen-activated protein kinase pathway was confirmed to be involved in the carvacrol-induced anti-proliferation and apoptosis in HepG-2 cells. These findings suggest that carvacrol may be a potential chemopreventive agent in cancer, and further experiments to investigate these possibilities are required.

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