Inhibition of cell proliferation by nobiletin, a dietary phytochemical, associated with apoptosis and characteristic gene expression, but lack of effect on early rat hepatocarcinogenesis *in vivo*

Hiroyuki Ohnishi,[1,2] Makoto Asamoto,[1,5] Kazunari Tujimura,[1,3] Naomi Hokaiwado,[1] Satoru Takahashi,[1] Kumiko Ogawa,[1] Masanori Kuribayashi,[1,4] Tadashi Ogiso,[1] Harumi Okuyama[2] and Tomoyuki Shirai[1]

[1]Department of Experimental Pathology and Tumor Biology, Nagoya City University, Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601; [2]Department of Preventive Nutraceutical Sciences, Nagoya City University, Graduate School of Pharmaceutical Sciences, 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467-8603; [3]Chemicals Evaluation and Research Institute, Chemicals Assessment Center, 1600 Shimotakano,Sugito-machi, Kitakatsushika-gun, Saitama 345-0043; [4]Ono Pharmaceuticals, Safety Research Institute, 1-5-2 Yamagishi Technoport, Mikuni-machi, Sakai-gun, Fukui 913-8538

(Received August 11, 2004/Revised October 12, 2004/Accepted October 19, 2004)

Dietary phytochemicals can inhibit the development of certain types of tumors. We here investigated the effects of nobiletin (Nob), garcinol (Gar), auraptene (Aur), β-cryptoxanthin- and hesperidine-rich pulp (CHRP) and 1,1′-acetoxychavicol acetate (ACA) on hepatocarcinogenesis in a rat medium-term liver bioassay, and also examined their influence on cell proliferation, cell cycle kinetics, apoptosis and cell invasion of rat and human hepatocellular carcinoma (HCC) cells, MH1C1 and HepG2, respectively. While there were no obvious suppressive effects on the development of putative preneoplastic liver lesions, inhibition of hepatocarcinoma cell proliferation was evident in the Nob group. Nob also caused G2/M cell cycle arrest and apoptosis. Microarray analysis identified a set of genes specifically regulated by Nob, and these are likely to be involved in the observed growth suppression of HCC cells. These results suggest that phytochemicals might have chemopreventive potential in late stages of hepatocarcinogenesis. (Cancer Sci 2004; 95: 936–942)

Dietary phytochemicals are attractive as chemopreventive agents for cancer development and there are a number of promising candidates. For instance, it has been shown that soybean isoflavones can inhibit experimental prostate cancer development *in vivo*[1] and *in vitro*[2,3] and intake of citrus fruits suppresses certain types of tumors.[4,5] Recent examples include nobiletin (Nob, a polymethoxyflavonoid in citrus fruits), garcinol (Gar, an antioxidant isolated from *Garcinia indica* fruit rind), auraptene (Aur, a Citrus antioxidant), β-cryptoxanthin- and hesperidine-rich pulp (CHRP, a powder containing large amounts of β-cryptoxanthin and hesperidine, prepared from a Satsuma mandarin juice) and 1,1′-acetoxychavicol acetate (ACA, present in seeds and rhizomes of *Languas galanga*, used as a ginger substitute and a stomach medicine in Thailand), which inhibit the development of several types of tumors.[6–11] When given orally they were found to reduce cancers of the tongue, esophagus and colon in rat experiments.[7,12,13] They also downregulated expression of cyclooxygenase-2 (COX-2) and nitric oxide synthase (NOS) in rat colon, with a concomitant decrease in inflammatory responses and oxidative stress, suggesting a potential for colon cancer inhibition.[7] Induction of detoxification enzymes and inhibition of cell proliferation[14,15] are also possible mechanisms of their suppressive effects.

Rat medium-term liver bioassays are effective to evaluate the effects of chemicals on hepatocarcinogenesis.[16] In our model, rats initiated with diethylnitrosamine (DEN) are treated with test chemicals by oral administration and pre-neoplastic lesions of the liver are measured by quantitation of immunohistochemically detected glutathione *S*-transferase placental form (GST-P)-positive foci in the liver,[17] with the help of an image analyzer.

In the present study, we focused on effects of a series of phytochemicals in this model and also investigated the influence of these compounds on the growth of a human HCC cell line, HepG2 and a rat HCC cell line, MH1C1, looking at cell proliferation and apoptosis. Moreover, we performed microarray analyses to identify changes in gene expression in HepG2 cells treated with these chemicals. We also investigated their effects on invasion of HCC cells using "Matrigel" invasion assays.

Materials and Methods

Cell culture and chemicals. Nobiletin (Nob), garcinol (Gar), auraptene (Aur), β-cryptoxanthin- and hesperidin-rich pulp (CHRP) and 1,1′-acetoxychavicol acetate (ACA) were gifts from Drs. T. Tanaka (Kanazawa Medical Univ.) and A. Murakami (Kinki Univ.). Their purity or composition was as follows: Nob, >98% purity; Gar, >98% purity; Aur, >98% purity; CHRP, 100 g prepared from a commercial mandarin juice, containing 1.1 g of water, 39.1 g of protein, 32.9 g of fat, 0.4 g of fiber, 1.4 g of ash, 0.67 g of β-cryptoxanthin, 3.58 g of hesperidin and 20.83 g of other compounds, including sugars, carotenoids other than β-cryptoxanthin, steroids, reduced vitamin C and unknown components; ACA, >95% purity. A human hepatocarcinoma cell line, HepG2, and a Morris hepatoma (#7795, Buffalo strain) cell line, MH1C1, were purchased from the American Type Culture Collection (Manassas, USA) and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS).[18]

Animals. Six-week-old male F344 rats were obtained from Charles River Japan, Inc. (Atsugi, Japan) and housed in plastic cages with hardwood chip bedding in an air-conditioned room at 23±2°C and 55±5% humidity with a 12 h light/dark cycle on basal diet (Oriental MF, Oriental Yeast Co., Tokyo) and tap water *ad libitum*.

Rat medium-term liver bioassay. Animals were randomly divided into six groups of 15 rats each. All groups received an intraperitoneal injection of DEN at a dose of 200 mg/kg b.w. as an initiation procedure. Starting 2 weeks thereafter, they were

[5]To whom correspondence should be addressed.
E-mail: masamoto@med.nagoya-cu.ac.jp

Abbreviations: ACA, 1,1′-acetoxychavicol acetate; Aur, auraptene; CHRP, β-cryptoxanthin- and hesperidine-rich pulp; DEN, diethylnitrosamine; EC_{20}, 20% effective concentration; Gar, garcinol; GST-P, glutathione *S*-transferase placental form; HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; Nob, nobiletin.

administered basal diet containing no supplement (Control group), or 500 ppm Nob, Gar, Aur, CHRP or ACA. All rats were subjected to two-thirds partial hepatectomy at the end of week 3. Body weights and food consumption were recorded twice a week and all surviving animals were killed under diethyl ether anesthesia at week 8. The livers were immediately excised, weighed and cut into 2- to 3-mm-thick slices, one from the caudate lobe and two from the right lateral lobe. The slices were fixed in ice-cold acetone for immunohistochemical visualization of GST-P-positive foci. The remaining livers were immediately frozen in liquid nitrogen and stored at −80°C until processed. The animal protocols were approved by the animal experimentation committee of Nagoya City University Graduate School of Medical Sciences.

Immunohistochemistry. Liver sections were exposed to rabbit anti-rat GST-P antibodies (MBL, Nagoya, Japan) and then sequentially to secondary antibody and avidin-biotin (Vectastain ABC Elite kit, Vector Laboratories, Inc., CA). The sites of peroxidase binding were demonstrated with diaminobenzidine as the substrate. Sections were then counterstained with hematoxylin for microscopic examination. Areas of GST-P-positive foci larger than 0.2 mm in diameter in the liver were quantitatively measured with an Image Processor for Analytical Pathology (IPAP-WIN, Sumika Technos Co., Osaka, Japan).

Determination of dose for the cell line experiments. The cell lines were preincubated at a density of 5×10^4 cells/ml (HepG2 cells) or 1×10^5 cells/ml (MH1C1 cells) on 6-well plates for 24 h. The culture medium was then replaced with fresh medium containing test chemicals at various concentrations or the vehicle (1% v/v DMSO), followed by incubation for a further 72 h. Cell survival was measured using a Cell Counting Kit (Dojin Laboratories, Kumamoto, Japan) with a sulfonated tetrazolium salt, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium salt monosodium salt (WST-1), according to the manufacturer's protocol.[19] The concentrations of the chemicals at which survival rates were less than 80% of the vehicle-treated control value (EC_{20}), were determined. This study was performed to examine chemopreventive effects, so it was important to avoid toxicity. Therefore, we selected EC_{20} or smaller doses for the assays of cell proliferation, cell cycle, apoptosis and cell invasion, as well as for the gene expression analysis.

Proliferation of HCC cells. HCC cell lines were preincubated in 6-well plates (2.5×10^5 cells for HepG2 cells and 4×10^5 for MH1C1). After 24 h incubation followed by medium refreshment, a test compound or the vehicle (1% v/v DMSO) was added to the cell culture medium. The medium was exchanged every 3 days. Cell numbers were measured at 1, 2, 3, 4, 6, 8 and 10 days and at 1, 2, 3, 4, 6 and 8 days of incubation for HepG2 and MH1C1cells, respectively, using a Coulter Counter (Beckman Coulter, Inc.).

Analysis of the cell cycle by flow cytometry. HCC cell lines were preincubated at a density of 5×10^4 cells/ml for HepG2 and 1×10^5 cells/ml for MH1C1 in culture flasks for 24 h. The culture medium was replaced with fresh aliquots containing test chemicals. After 72 h incubation the cells were trypsinized, washed three times with ice-cold phosphate-buffered saline containing 1% FBS, resuspended in 1 ml of 0.1% Triton X-100 and mixed with 1 ml of 10 mg/ml RNase solution. Then, the cells were stained with 50 µl of 1 mg/ml propidium iodide and the cell cycle was analyzed with a cell cycle analyzer, Guava PCA (Guava Technologies, Inc., USA) and Multicycle AV software for Windows (Phoenix Flow Systems).

Detection of apoptotic cell death. For detection of apoptotic cells, annexin assays were performed with a Guava Nexin kit (Guava Technologies, Inc.) according to the manufacturer's protocol. Briefly, HepG2 cells and MH1C1 cells were treated with chemicals for 72 h, trypsinized and washed three times with 1 ml of Nexin buffer. They were then centrifuged for collection and resuspended in 50 µl of Nexin buffer. Aliquots of 5 µl of Annexin áX-PE and 5 µl of 7-AAD were added to 40 µl aliquots, and the mixtures were incubated on ice for 20 min under shielding from light, then analyzed with Guava PCA (Guava Technologies, Inc.).

Microarray analysis. HepG2 cells were cultured for 24 h at a density of 5×10^4 cells/ml. After 24 h preincubation, the culture medium was replaced with fresh medium containing a test chemical. After culture for 72 h, cells were trypsinized and cellular RNA was extracted using ISOGEN (Nippon Gene, Toyama, Japan). Microarray analyses were performed using Acegene human oligo chip subset A (containing about 10,000 genes, Hitachi Software Engineering, Inc., Tokyo) according to the manufacturer's protocol, and data were confirmed in duplicate tests. Normalization of gene expression was achieved by using a mean±2SD restriction. Accuracy for each gene (CV score; CV=SD×100/average) was measured and genes for which CV scores were <-40 or >40 were selected as valid.

Real-time semi-quantitative PCR (RT-PCR). To confirm the results of microarray analysis, we performed semi-quantitative RT-PCR with chemical-treated HepG2 cells using a Light cycler (Roche Diagnostics Japan, Tokyo) according to the manufacturer's instructions. We also measured the expression of genes whose functions were related to the cell cycle or apoptosis in Nob-treated HepG2 cells. Data were expressed relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Primers used in this assay are listed in Table 1.

Invasion assay. Invasion activities of HCC cells were assessed in an invasion assay using BD "Biocoat" "Matrigel" invasion chambers (BD Biosciences) according to the manufacturer's protocol and after Saito *et al.*[20]. Briefly, HepG2 or MH1C1 cells were cultured on the upper surfaces of invasion chambers at a density of 1×10^5 cells/ml in medium containing hepatocyte growth factor (HGF, Wako Chemicals) at 10 ng/ml and 100 ng/ml for HepG2 and MH1C1 cells, respectively, and the phytochemicals were added to the lower wells. After 22 h incubation, chambers were fixed with methanol and cells that had invaded the lower surfaces were stained with 0.5% crystal violet in 20% methanol for 30 min. The upper surfaces of the filters were rinsed with water, and cells were removed with a cotton swab. The filters were transferred to separate wells in 96-well culture plates. Crystal violet dye retained on the filters was extracted with 30% acetic acid and the absorbance was colorimetrically measured at 590 nm.

Statistical evaluation. In the *in vivo* study, data were analyzed

Table 1. Primers used for quantitative RT-PCR analysis

	Upper primer	Lower primer
CDK2	cacagctgtggacatctgga	gtgcagcatttgcgataaca
CDK1 (cdc2)	cttttccatggggattcaga	ccattttgccagaaattcgt
Cyclin B1	cctgagcctattttggttga	tcccgacccagtaggtattt
P21	gaccatgtggacctgtcact	cggccagggtatgtacatga
E2F1	ctcattgccaagaagtccaa	ctcagggcacaggaaaacat
14-3-3-σ	gccctgaacttttccgtctt	cgtccacagtgtcaggttgt
Bcl-2	tgtggccttctttgagttcg	cttcacttgtggcccagata
Bcl-xL	tgttttggacaatggactgg	tgggatgtcaggtcactgaa
VDAC1	agacagggtacaagcgggag	cagcggtctccaacttcttg
Caspase-9	cgtggtggtcattctctctc	ctggatgaaaaagagcttgg
Csk	ctttgagaacctcatgcagc	cgactttgttccctcggtaa
IGF1R	ggcaatttgctcattaacat	aagttctggttgtcgaggac
Caspase-2	gcctgtcgacagatactgtg	gcgaaattccagttctttct
RXRα	catctttgacagggtgctga	ctgggtacttgtgcttgcag
Cyclin E2	agccgtttacaagctaagca	ataatgcaaggactgatccc
PCNA	atgccttctggtgaatttgc	tcaccgttgaagagagtgga
GAPDH	aaggtcggagtcaacggatt	ttgccatgggtggaatcata

Table 2. Body and relative organ weight at the end of study

Group	No. of rats examined	Body weight (g)	Liver weight (g)	Kidney weight (g)	Right-kidny weight (g)	Left-kidney weight (g)	Relative liver weight (%)	Relative kidney weight (%)
Control	13	275±11	8.67±0.58	1.75±0.10	0.87±0.05	0.88±0.05	3.14±0.18	0.64±0.03
Nobiletin	15	274±13	8.92±0.66	1.92±0.13	0.92±0.19	1.00±0.13	3.25±0.13	0.70±0.04
Garcinol	16	278±10	8.66±0.42	1.92±0.12	0.96±0.06	0.96±0.07	3.11±0.13	0.69±0.04
Auraptene	16	269±13	8.63±0.59	1.86±0.13	0.93±0.07	0.93±0.07	3.20±0.11	0.69±0.04
CHRP	16	278±11	8.58±0.40	1.77±0.08	0.89±0.05	0.89±0.04	3.09±0.09	0.64±0.02
ACA	15	275±14	8.73±0.67	1.77±0.12	0.88±0.06	0.90±0.07	3.17±0.13	0.65±0.02

Data are expressed as means±SD. Statistical analysis followed by Kruskal-Wallis test.

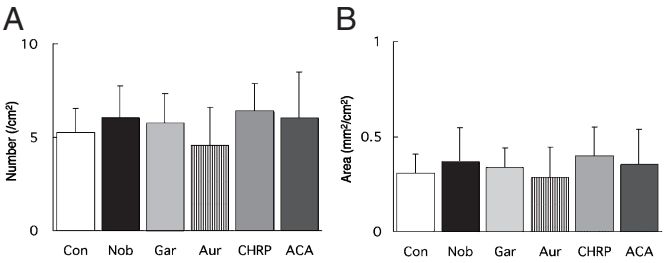

Fig. 1. Evaluation of liver GST-P-positive foci. (A) Numbers of GST-P-positive foci per unit area in the livers of rats treated with chemicals. (B) Areas of GST-P-positive foci per unit area. Statistical analysis was performed with the Kruskal-Wallis test.

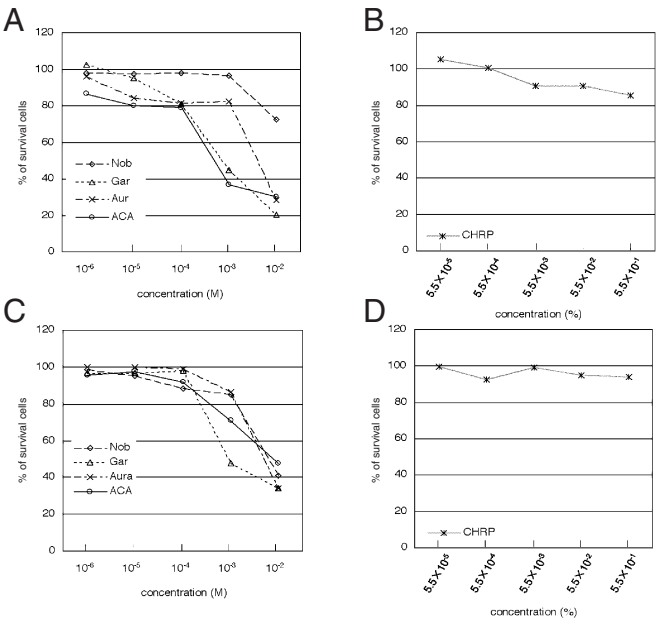

Fig. 2. EC_{20} determination for chemicals with HepG2 cells (A and B) and MH1C1 cells (C and D) compared to vehicle-treated controls. Each cell line was treated with chemicals, and the number of cells was measured by WST-1 assay after 3 days culture. The CHRP concentration was expressed as a percentage compared to control cells, because CHRP is a mixture of chemicals.

by applying the Kruskal-Wallis test. In the *in vitro* study, statistical analysis was performed with the Bonferroni/Dunn test. P values of less than 0.05 were considered to indicate significant differences.

Results

Evaluation of rat liver GST-P-positive foci. In the rat medium-term liver bioassay,[16] body weights, food intake and relative organ weights in the experimental period did not differ among the groups (Table 2) and there was no significant variation in the numbers and areas of GST-P-positive foci (Fig. 1).

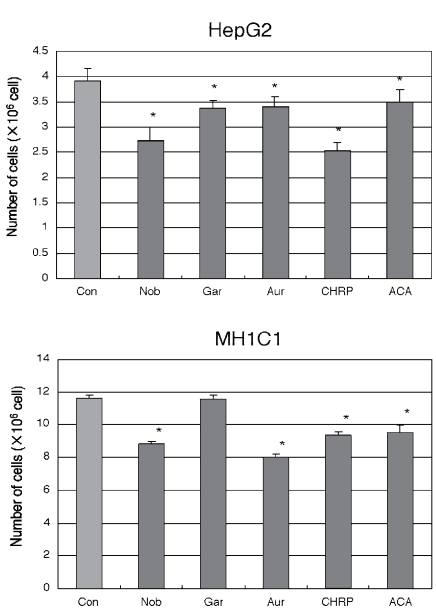

Fig. 3. Effects of chemicals on HepG2 (A) and MH1C1 (B) cell proliferation. These graphs show the numbers of cells treated for 8 days with the chemicals. All treated cells, except MH1C1 cells treated with garcinol, showed reduced proliferation compared to the non-treated cells.

Determination of doses for the cell line experiments. As shown in Fig. 2, dose-related decreases in cell number were observed with all chemical treatments. From the data, we determined doses for HepG2 cells of: Nob, 10^{-3} *M*; Gar, 10^{-4} *M*; Aur, 10^{-3} *M*; CHRP, 5.5×10^{-1}%; ACA, 10^{-5} *M* (Fig. 2). For MH1C1 cells, the corresponding values were: Nob, 10^{-3} *M*; Gar, 10^{-4} *M*; Aur, 10^{-3} *M*; CHRP, 5.5×10^{-1}%; ACA, 10^{-4} *M* (Fig. 2). These concentrations of the chemicals for each cell line were employed for further *in vitro* assays.

Effects of chemicals on HCC cell proliferation. The experiment was repeated five times. All phytochemicals significantly reduced proliferation of HepG2 cells (Fig. 3A), and also MH1C1 cells (Fig. 3B), with the exception of garcinol. Statistical analysis was performed with the Bonferroni/Dunn test and the values were significantly different ($P<0.01$) from those of vehicle-treated control cells.

Effect of chemicals on cell cycle kinetics. After 3 days exposure, cell cycle kinetics of HCC cell lines were analyzed (Fig. 4). With MH1C1 cells, Nob significantly increased the amounts of G2- or M-phase cells and decreased the proportion of S-phase cells. With HepG2 cells, Nob also significantly increased the cells in G2- or M-phase.

Apoptosis induction in HCC cell lines. The annexin assay revealed that Nob, Aur and ACA significantly increased total apoptosis of HepG2 cells. Nob and Aur also increased the number of early apoptotic HepG2 cells. Furthermore, Nob increased late and total apoptotic MH1C1 cells (Fig. 5).

Gene expression profile of HepG2 cells. As shown in Fig. 6, we selected a gene set unique to Nob using cluster analysis, because only this compound inhibited cell cycle progression and

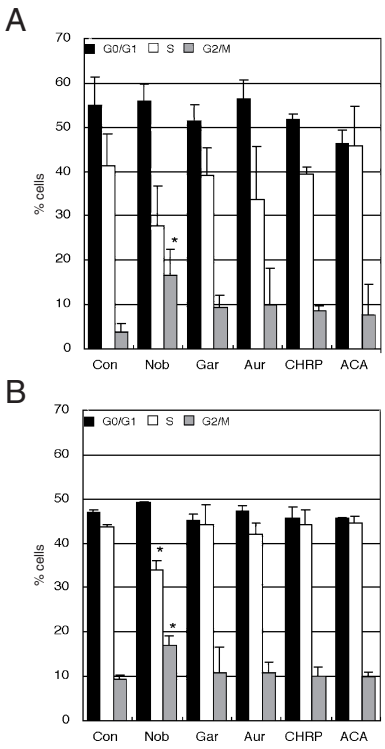

Fig. 4. Cell cycle kinetics of chemical-treated HepG2 cells (A) and MH1C1 cells (B). 5×10^4 HepG2 and 1×10^5 MH1C1 cells/ml were seeded in culture flasks. After 3 days exposure to chemicals, the cell cycle kinetics were analyzed by flow cytometry. Data are means±SD. This experiment was repeated four times. Statistical analysis was performed with the Bonferroni/Dunn test and asterisks indicate a significant difference ($P<0.01$) compared to vehicle-treated control cells.

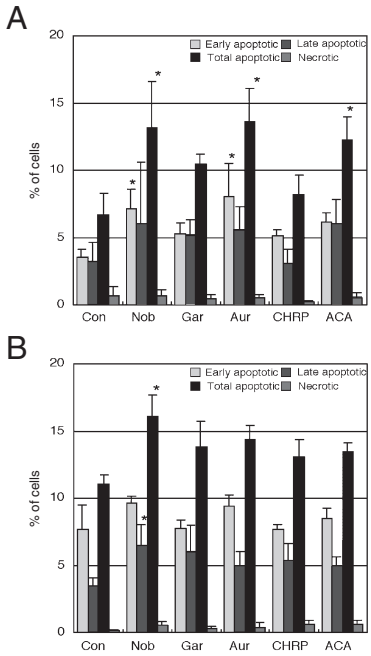

Fig. 5. Apoptotic cell death of chemical-treated HepG2 (A) and MH1C1 (B) cells. 5×10^4 HepG2 and 1×10^5 MH1C1 cells/ml were seeded in culture flasks. After 3 days exposure to chemicals, apoptosis was evaluated by means of Annexin assays. Data are expressed as means±SD. This experiment was repeated four times. Statistical analysis was performed with the Bonferroni/Dunn test and asterisks indicate a significant difference ($P<0.01$) compared with vehicle-treated control cells.

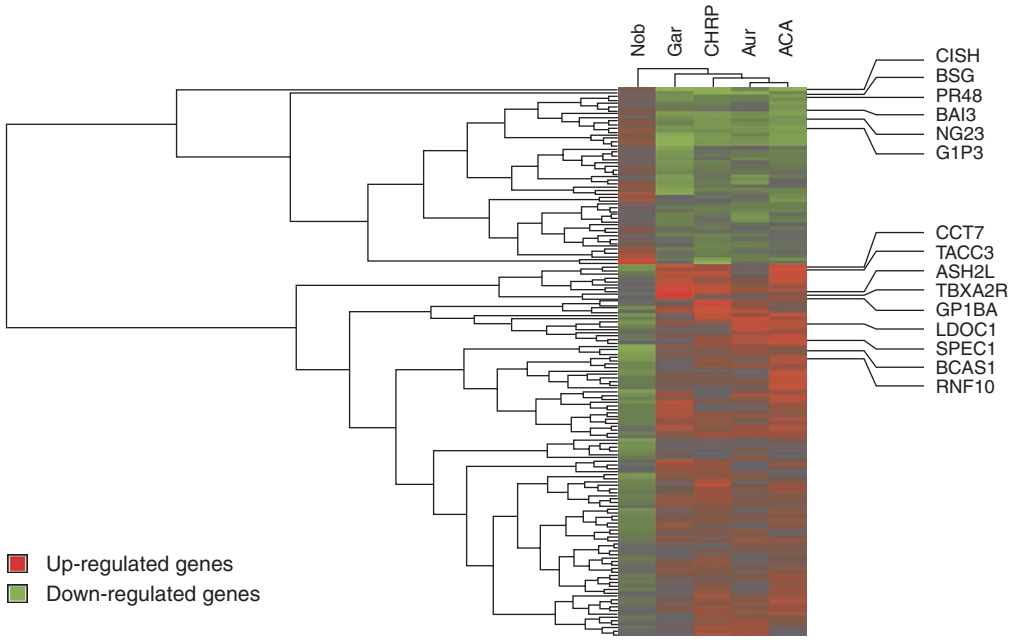

Fig. 6. Clustering analysis of microarray results for chemical-treated HepG2 cells. Total RNAs were used in this assay. The cluster represents Nob-specific regulated genes.

induced apoptosis. We focused on genes that were related to the cell cycle, apoptosis, cell proliferation or signal transduction (Table 3) using the "Gene Ontology" functional classification of genes.

Expression of cell cycle- or apoptosis-related genes. We performed real-time semi-quantitative RT-PCR to confirm changes in expression of the proliferated cell nuclear antigen (PCNA) gene in HepG2 cells treated with chemicals and found a good correlation with microarray results (Fig. 7). Real-time semi-quantitative RT-PCR for HepG2 and MH1C1 cells treated with Nob was further conducted to estimate changes in genes related to the cell cycle or apoptosis (Fig. 8). As a result, the 14-3-3-σ gene was found to be down-regulated, while the voltage dependent anion channel 1 (VDAC1) gene and the retinoid X receptor α (RXRα) gene were upregulated in Nob-treated HepG2 cells. The cyclin E2, bcl-2 and cyclin B1 genes were downregulated, and the 14-3-3 σ gene was upregulated in Nob-treated MH1C1 cells.

Invasive activities of HCC cells treated with chemicals. "Matrigel" Invasion assays revealed that Gar, Aur and CHRP treatments reduced HGF-induced cell invasion by HepG2 and MH1C1 cells, but the effect was not statistically significant (Fig. 9).

Discussion

While the present investigation of a series of phytochemicals revealed no modifying effects on the development of rat liver GST-P-positive foci in a medium-term model, a clear influence on the growth of HepG2 and MH1C1 cell lines was evident at the EC_{20} concentration. In particular, Nob induced G2/M cell cycle arrest of both HCC cells. It was also found that Nob, Aur

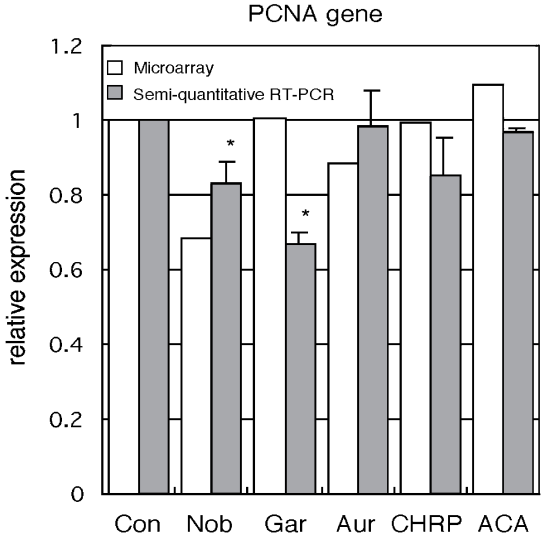

Fig. 7. Semi-quantitative RT-PCR analysis of the PCNA gene, relative to expression of the GAPDH gene. This experiment was reproduced three times. Statistical analysis was performed with the Bonferroni/Dunn test and asterisks indicate a significant difference ($P<0.01$) compared with vehicle-treated control cells.

Table 3. Signal transduction-related and Nob-specific regulated genes in HepG2 cells

Upregulated by Nob	
CISH	Cytokine-inducible sh2-containing protein
BSG	Basigin
PR48	Protein phosphatase 2A 48 kDa regulatory subunit
BAI3	Brain-specific angiogenesis inhibitor 3
NG23	Ng23 protein
G1P3	Interferon induced 6-16 protein, isoform b
Downregulated by Nob	
CCT7	Chaperonin containing tcp1, subunit 7 (eta)
TACC3	Transforming acidic coiled-coil containing protein 3
ASH2L	Ash2 (absent, small, or homeotic)-like (*Drosophila*)
TBXA2R	Thromboxane a2 receptor
GP1BA	Platelet glycoprotein ib α polypeptide precursor
LDOC1	Leucine zipper, downregulated in cancer 1
SPEC1	Small protein effector 1 of cdc42
BCAS1	Breast carcinoma amplified sequence 1
RNF10	Ring finger protein 10

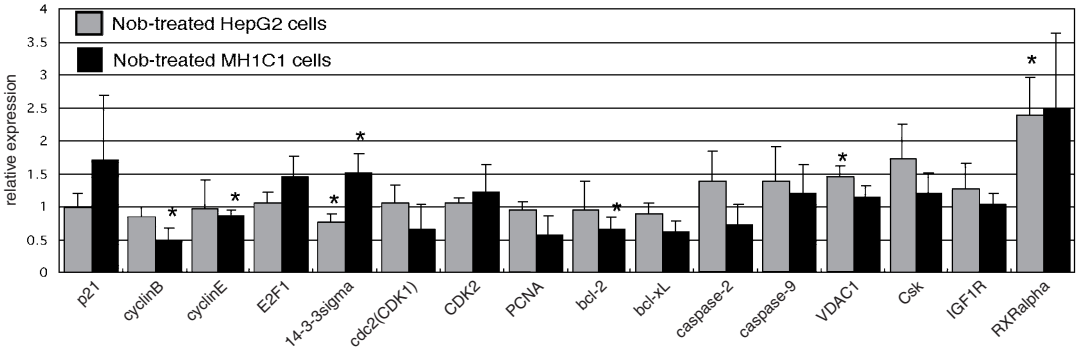

Fig. 8. Semi-quantitative RT-PCR analysis of cell cycle- or apoptosis-related genes in Nob-treated HepG2 and MH1C1 cells. Expression of genes is relative to that of the GAPDH gene. This experiment was repeated three times. Statistical analysis was performed with the Bonferroni/Dunn test and asterisks indicate a significant difference ($P<0.01$) compared to vehicle-treated control cells.

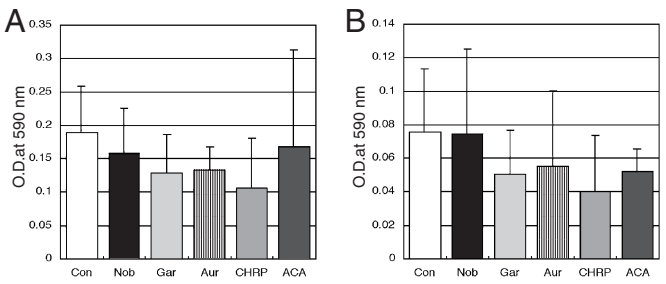

Fig. 9. Effect of chemicals on HGF-induced HepG2 (A) and MH1C1 (B) cell invasion, analyzed with "Biocoat" "Matrigel" invasion chambers. 1×10^5 cell/ml cells were seeded in each chamber and 10 or 100 ng/ml HGF was added to the cultures of HepG2 and MH1C1 cells, respectively. After 24 h treatment with chemicals, cells that had invaded were stained with crystal violet, which was then measured by spectrometry at 590 nm. This assay was repeated five times. Statistical analysis was performed with the Bonferroni/Dunn test.

and ACA induced apoptosis. Furthermore, Gar, Aur and CHRP suppressed HGF-induced cell invasion to some extent. There is a discrepancy between our *in vivo* and *in vitro* data. However, in the *in vivo* study, we analyzed only preneoplastic GST-P-positive foci. For the *in vitro* studies, we used cancer cells (hepatomas). Recently, the results of a long-term *in vivo* study were published, showing Aur to inhibit late stages of liver carcinogenesis.[21] Therefore, our *in vivo* and *in vitro* data presented in this paper appear reasonable. Thus, the phytochemicals applied in this study showed suppressive effects on the growth of HCC cells while not causing any significant modification of early hepatocarcinogenesis.

We cannot exclude the possibility that the EC_{20} doses of the chemicals made HCC cells necrotic. However, Nob appeared to have inhibitory effects against the HCC cell line, since it induced G2/M cell cycle arrest and caused apoptosis. Aur and ACA also induced apoptosis. Growth-inhibitory effects of Aur and ACA have been reported, but the reasons for this and the mechanisms of apoptosis induction remain unknown. Induction of phase II enzymes by these chemicals has been reported, but the mechanisms of chemoprevention need to be precisely identified. In our medium-term liver bioassay, these compounds did not inhibit the development of GST-P foci. However recently, another group reported that Aur inhibits rat liver carcinogenesis in a longer experimental regimen.[21] Compounds which induce apoptosis and cell cycle arrest *in vitro* clearly might be expected to exert chemopreventive effects. Furthermore, analysis of gene expression profiles of chemical-treated HepG2 cells established a set of genes which were specifically regulated by Nob and clearly could be related to its inhibition of HepG2 cell proliferation and induction of apoptosis and G2/M cell cycle arrest. Products of signal transduction-related genes upregulated by Nob treatment included cytokine-inducible SH2-containing protein (CISH), basigin (BSG), and the protein phosphatase 2A 48 kDa regulatory subunit (PR48). Genes downregulated by Nob encoded chaperonin TCP1 subunit 7 (eta) (CCT7), transforming acid coiled-coil containing protein 3 (TACC3), and ash2 (absent, small or homeotic)-like (*Drosophila*) (ASH2L). CISH is cytokine-regulator gene and suppressor of JAK-STAT signal transduction.[22] No role for this gene in hepatocellular carcinomas has hitherto been reported, but the SOCS-1 gene, belonging to the same family, has been shown to be methylated, with reduced activity, in hepatocellular carcinoma.[23] BSG, also called EMMPRIN, induces matrix metalloproteinase and contributes to cell invasion.[24] PR48 is the regulatory subunit of protein tyrosine phosphatase 2a and overexpression of this gene is known to cause cell cycle arrest.[25] The CCT7 gene is related to the cell cycle,[26] but its function in hepatocytes or HCCs has not been clarified. TACC3 is related to cell growth and differentiation, and this gene has been suggested to be upregulated in some cancers.[27] No role of ASH2L has hitherto been suggested in hepatocytes, but downregulation is associated with megakaryocyte differentiation.[28] Suppression of colon carcinogenesis by some phytochemicals used in this study was suggested to be mediated by reduction in cyclooxygenase-2 expression and inhibition of colon inflammatory responses.[7] In this study, however, there were no specific alterations of genes related to inflammation. One type of genes related to inflammation is cytokine-inducible, but in this study their expression was not affected. The chemicals in this study have been shown to induce phase II enzymes. However, expression of genes impacting on drug metabolism, such as those encoding cytochrome P450, glutathione *S*-transferase and quinone reductase in liver, was also not appreciably changed.[14]

Real-time semi-quantitative RT-PCR revealed downregulation of cell cycle-related genes, cyclin E, cyclin B and bcl-2 in Nob-treated MH1C1 cells. Since cyclin B1 plays a major role in G2/M cell cycle progression, reduction in its gene expression is indicative of G2/M cell cycle arrest. Reduction of bcl-2 gene expression is an established cause of apoptosis. Upregulation of retinoid X receptor α (RXRα) has been suggested to inhibit AP-1 activity[29] and its degradation has been demonstrated in HCC.[30] It has also been suggested that an acyclic retinoid, a ligand of RXRα, induces apoptosis and inhibits HCC growth.[31] Moreover, a clinical study showed it to suppress HCC relapse.[32] In this study, although we did not investigate RXRα degradation, upregulation of gene expression might have a role in the inhibition of HCC cell proliferation by Nob. However, these genes related to signal transduction are also regulated by phosphorylation or at the translational level, so evaluation of protein expression is also on the agenda for the future.

In conclusion, the present study demonstrated chemopreventive potential of dietary phytochemicals against growth of HCC cell lines. In particular, Nob treatment suppressed HCC cell growth by inducing cell cycle inhibition and apoptosis. Furthermore, microarray analysis demonstrated changes in a set of genes related to cell growth in Nob-treated HepG2 cells. While the dietary phytochemicals used in this study had no apparent effects on early stages of *in vivo* hepatocarcinogenesis, they exerted an inhibitory influence on HCC cell growth that warrants further exploration.

This research was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan, a Grant-in-Aid for the 2nd Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare of Japan, and a grant from the Society for Promotion of Pathology of Nagoya, Japan.

1. Choi YH, Lee WH, Park KY, Zhang L. p53-independent induction of p21 (WAF1/CIP1), reduction of cyclin B1 and G2/M arrest by the isoflavone genistein in human prostate carcinoma cells. *Jpn J Cancer Res* 2000; **91**: 164–73.
2. Bosland MC, McCormick DL, Melamed J, Walden PD, Zeleniuch-Jacquotte A, Lumey LH. Chemoprevention strategies for prostate cancer. *Eur J Cancer Prev* 2002; **11** Suppl 2: S18–27.
3. Hikosaka A, Asamoto M, Hokaiwado N, Kato K, Kuzutani K, Kohri K, Shirai T. Inhibitory effects of soy isoflavones on rat prostate carcinogenesis induced by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). *Carcinogenesis* 2004; **25**: 381–7.
4. Kohno H, Taima M, Sumida T, Azuma Y, Ogawa H, Tanaka T. Inhibitory effect of mandarin juice rich in beta-cryptoxanthin and hesperidin on 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced pulmonary tumorigenesis

in mice. *Cancer Lett* 2001; **174**: 141–50.
5. Kossoy G, Ben-Hur H, Stark A, Zusman I, Madar Z. Effects of a 15% orange-pulp diet on tumorigenesis and immune response in rats with colon tumors. *Oncol Rep* 2001; **8**: 1387–91.
6. Murakami A, Nakamura Y, Torikai K, Tanaka T, Koshiba T, Koshimizu K, Kuwahara S, Takahashi Y, Ogawa K, Yano M, Tokuda H, Nishino H, Mimaki Y, Sashida Y, Kitanaka S, Ohigashi H. Inhibitory effect of citrus nobiletin on phorbol ester-induced skin inflammation, oxidative stress, and tumor promotion in mice. *Cancer Res* 2000; **60**: 5059–66.
7. Tanaka T, Kohno H, Shimada R, Kagami S, Yamaguchi F, Kataoka S, Ariga T, Murakami A, Koshimizu K, Ohigashi H. Prevention of colonic aberrant crypt foci by dietary feeding of garcinol in male F344 rats. *Carcinogenesis* 2000; **21**: 1183–9.
8. Tanaka T, Kawabata K, Kakumoto M, Makita H, Hara A, Mori H, Satoh K, Murakami A, Kuki W, Takahashi Y, Yonei H, Koshimizu K, Ohigashi H. Citrus auraptene inhibits chemically induced colonic aberrant crypt foci in male F344 rats. *Carcinogenesis* 1997; **18**: 2155–61.
9. Narisawa T, Fukaura Y, Oshima S, Inakuma T, Yano M, Nishino H. Chemoprevention by the oxygenated carotenoid beta-cryptoxanthin of *N*-methylnitrosourea-induced colon carcinogenesis in F344 rats. *Jpn J Cancer Res* 1999; **90**: 1061–5.
10. Tanaka T, Makita H, Kawabata K, Mori H, Kakumoto M, Satoh K, Hara A, Sumida T, Ogawa H. Chemoprevention of azoxymethane-induced rat colon carcinogenesis by the naturally occurring flavonoids, diosmin and hesperidin. *Carcinogenesis* 1997; **18**: 957–65.
11. Tanaka T, Kawabata K, Kakumoto M, Makita H, Matsunaga K, Mori H, Satoh K, Hara A, Murakami A, Koshimizu K, Ohigashi H. Chemoprevention of azoxymethane-induced rat colon carcinogenesis by a xanthine oxidase inhibitor, 1′-acetoxychavicol acetate. *Jpn J Cancer Res* 1997; **88**: 821–30.
12. Tanaka T, Kawabata K, Kakumoto M, Matsunaga K, Mori H, Murakami A, Kuki W, Takahashi Y, Yonei H, Satoh K, Hara A, Maeda M, Ota T, Odashima S, Koshimizu K, Ohigashi H. Chemoprevention of 4-nitroquinoline 1-oxide-induced oral carcinogenesis by citrus auraptene in rats. *Carcinogenesis* 1998; **19**: 425–31.
13. Kawabata K, Tanaka T, Yamamoto T, Hara A, Murakami A, Koshimizu K, Ohigashi H, Stoner GD, Mori H. Suppression of N-nitrosomethylbenzylamine-induced rat esophageal tumorigenesis by dietary feeding of auraptene. *J Exp Clin Cancer Res* 2000; **19**: 45–52.
14. Tanaka T, Kawabata K, Kakumoto M, Hara A, Murakami A, Kuki W, Takahashi Y, Yonei H, Maeda M, Ota T, Odashima S, Yamane T, Koshimizu K, Ohigashi H. Citrus auraptene exerts dose-dependent chemopreventive activity in rat large bowel tumorigenesis: the inhibition correlates with suppression of cell proliferation and lipid peroxidation and with induction of phase II drug-metabolizing enzymes. *Cancer Res* 1998; **58**: 2550–6.
15. Zheng Q, Hirose Y, Yoshimi N, Murakami A, Koshimizu K, Ohigashi H, Sakata K, Matsumoto Y, Sayama Y, Mori H. Further investigation of the modifying effect of various chemopreventive agents on apoptosis and cell proliferation in human colon cancer cells. *J Cancer Res Clin Oncol* 2002; **128**: 539–46.
16. Ito N, Tamano S, Shirai T. A medium-term rat liver bioassay for rapid *in vivo* detection of carcinogenic potential of chemicals. *Cancer Sci* 2003; **94**: 3–8.
17. Ito N, Tsuda H, Tatematsu M, Inoue T, Tagawa Y, Aoki T, Uwagawa S, Kagawa M, Ogiso T, Masui T *et al.* Enhancing effect of various hepatocarcinogens on induction of preneoplastic glutathione S-transferase placental form positive foci in rats-an approach for a new medium-term bioassay system. *Carcinogenesis* 1998; **9**: 387–94.
18. Richardson UI, Tashjian AH Jr, Levine L. Establishment of a clonal strain of hepatoma cells which secrete albumin. *J Cell Biol* 1969; **40**: 236–47.
19. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; **65**: 55–63.
20. Saito K, Oku T, Ata N, Miyashiro H, Hattori M, Saiki I. A modified and convenient method for assessing tumor cell invasion and migration and its application to screening for inhibitors. *Biol Pharm Bull* 1997; **20**: 345–8.
21. Sakata K, Hara A, Hirose Y, Yamada Y, Kuno T, Katayama M, Yoshida K, Zheng Q, Murakami A, Ohigashi H, Ikemoto K, Koshimizu K, Tanaka T, Mori H. Dietary supplementation of the citrus antioxidant auraptene inhibits N,N-diethylnitrosamine-induced rat hepatocarcinogenesis. *Oncology* 2004; **66**: 244–52.
22. Jiang C, Yu L, Zhao Y, Zhang M, Liu Q, Mao N, Geng Z, Zhao S. Cloning and characterization of CIS 1b (cytokine inducible SH2-containing protein 1b), an alternative splicing form of the CIS 1 gene. *DNA Seq* 2000; **11**: 149–54.
23. Okochi O, Hibi K, Sakai M, Inoue S, Takeda S, Kaneko T, Nakao A. Methylation-mediated silencing of SOCS-1 gene in hepatocellular carcinoma derived from cirrhosis. *Clin Cancer Res* 2003; **9**: 5295–8.
24. Caudroy S, Polette M, Nawrocki-Raby B, Cao J, Toole BP, Zucker S, Birembaut P. EMMPRIN-mediated MMP regulation in tumor and endothelial cells. *Clin Exp Metastasis* 2002; **19**: 697–702.
25. Yan Z, Fedorov SA, Mumby MC, Williams RS. PR48, a novel regulatory subunit of protein phosphatase 2A, interacts with Cdc6 and modulates DNA replication in human cells. *Mol Cell Biol* 2000; **20**: 1021–9.
26. Miklos D, Caplan S, Mertens D, Hynes G, Pitluk Z, Kashi Y, Harrison-Lavoie K, Stevenson S, Brown C, Barrell B *et al.* Primary structure and function of a second essential member of the heterooligomeric TCP1 chaperonin complex of yeast, TCP1 beta. *Proc Natl Acad Sci USA* 1994; **91**: 2743–7.
27. Still IH, Vince P, Cowell JK. The third member of the transforming acidic coiled coil-containing gene family, TACC3, maps in 4p16, close to translocation breakpoints in multiple myeloma, and is upregulated in various cancer cell lines. *Genomics* 1999; **58**: 165–70.
28. Wang J, Zhou Y, Yin B, Du G, Huang X, Li G, Shen Y, Yuan J, Qiang B. ASH2L: alternative splicing and downregulation during induced megakaryocytic differentiation of multipotential leukemia cell lines. *J Mol Med* 2001; **79**: 399–405.
29. Salbert G, Fanjul A, Piedrafita FJ, Lu XP, Kim SJ, Tran P, Pfahl M. Retinoic acid receptors and retinoid X receptor-alpha down-regulate the transforming growth factor-beta 1 promoter by antagonizing AP-1 activity. *Mol Endocrinol* 1993; **7**: 1347–56.
30. Matsushima-Nishiwaki R, Shidoji Y, Nishiwaki S, Yamada T, Moriwaki H, Muto Y. Aberrant metabolism of retinoid X receptor proteins in human hepatocellular carcinoma. *Mol Cell Endocrinol* 1996; **121**: 179–90.
31. Nakamura N, Shidoji Y, Moriwaki H, Muto Y. Apoptosis in a human hepatoma cell line induced by 4,5-didehydrogeranylgeranoic acid (acyclic retinoid) via down-regulation of transforming growth factor-alpha. *Biochem Biophys Res Commun* 1996; **219**: 100–4.
32. Muto Y, Moriwaki H, Saito A. Prevention of second primary tumors by an acyclic retinoid in patients with hepatocellular carcinoma. *N Engl J Med* 1999; **340**: 1046–7.