Molecular mechanisms of the chemopreventive effect on hepatocellular carcinoma development in Mdr2 knockout mice

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Abstract

Dietary antioxidants and selenium compounds were shown to have a therapeutic effect against hepatocellular carcinoma in several mouse models. We tested the effects of tannic acid and selenomethionine on hepatocellular carcinoma development in Mdr2 knockout (Mdr2-KO) mice. Mdr2-KO and age-matched Mdr2 heterozygous control mice were fed with tannic acid or selenomethionine during the first 3 months of life. Then, several mice from each group were sacrificed, and liver tissue samples were removed for analysis. The remaining mice were fed a regular diet until the age of 16 months, at which time the number and size of liver tumors were determined. Liver tissue samples of 3-month-old mice were subjected to gene expression profiling analysis using cDNA macroarrays containing probes for 240 genes that regulate responses to oxidative stress and inflammation or lipid metabolism. Both tannic acid and selenomethionine had partial chemopreventive effect on development of hepatocellular carcinoma in Mdr2-KO mice: they reduced the incidence of large tumor nodules (diameter > 1 cm) at age 16 months. Both agents inhibited gene expression and reversed up-regulation of many genes that control inflammation or response to oxidative stress in Mdr2-KO livers at age 3 months. This inhibitory effect on gene expression correlated with the ability of agents to reduce incidence of large tumors: selenomethionine was more active than tannic acid in both aspects. Understanding the molecular mechanism of chemoprevention effect could improve our therapeutic modalities while using these agents. [Mol Cancer Ther 2007;6(4):1283–91]

Introduction

Mouse models of hepatocellular carcinoma have been widely used to study the molecular mechanisms of primary liver cancer (1–3), and it was recently shown that most mouse hepatocellular carcinomas are similar to specific subgroups of human hepatocellular carcinomas in terms of global gene expression patterns (4). We are studying the molecular mechanisms of hepatocarcinogenesis on Mdr2 knockout (Mdr2-KO) mice, a model for an inflammation-associated hepatocellular carcinoma (5–7). The Mdr2-KO mice lack the liver-specific P-glycoprotein responsible for phosphatidylcholine transport across the bile canalicular membrane (8). The absence of phospholipids from bile results in bile regurgitation (9) and portal inflammation followed by the development of hepatocyte dysplasia and hepatocellular carcinoma (5). We have recently shown induction of multiple protective mechanisms in the livers of Mdr2-KO mice at 3 months of age, particularly, the induction of antioxidant protective systems (7). We hypothesized that this induction could be caused by an oxidative stress at an early age, produced either directly by leaking bile, or indirectly by infiltrating macrophages and neutrophils. To test the effect of inflammation and suggested oxidative stress at an early age (3 months) on hepatocellular carcinoma development at a later age (16 months) in this model, we treated Mdr2-KO mice with antioxidant and anti-inflammatory agent tannic acid or with chemopreventive selenium compound selenomethionine from prenatal period until the age of 3 months.

Previously, antioxidant supplementation was shown to be beneficial against hepatocellular carcinoma development in several mouse models for hepatocellular carcinoma: chemoprevention was shown with vitamin E (10), selenium (11, 12), and tannic acid (13). Tannic acid is a potent antioxidant with multiple additional activities. It suppresses growth of cholangiocarcinoma by inhibiting cell cycle progression and increasing expression of the cyclin-dependent kinase inhibitor $p27^{KIP1}$ (14). It also inhibits tumor cell proteasomal activity and induces Bax expression and apoptosis (15). Tannic acid is a selective CXCL12/CXCR4

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antagonist with antiangiogenic and anti-inflammatory properties (16). It inhibits generation of an induced nitric oxide in rat hepatocytes (17) and attenuates plasminogen activator activity in the liver of C3H hepatocellular carcinoma model mice (18). Selenium is one of the most efficient chemopreventive agents. In animal cancer models, selenomethionine and selenocysteine are the most efficient chemopreventive selenium compounds (19). In addition to its role as an essential component of two antioxidant enzymes (thioredoxin reductase and glutathione peroxidase), selenium affects many cellular regulatory pathways (20, 21). Thus, for both tannic acid and selenomethionine, their chemopreventive activity on hepatocellular carcinoma could be attributed not only to an increase in antioxidant protection systems in the liver but also to their effects on the immune system, angiogenesis, and cell proliferation.

To explore the molecular mechanisms of tannic acid and selenomethionine action on Mdr2-KO mice, we did gene expression profiling in liver tissues of these mice at the end of treatment period (3 months) using cDNA macroarrays designed and manufactured in our laboratory. These arrays carry probes to genes that regulate lipid metabolism, angiogenesis, or responses to oxidative stress and inflammation. We have found that both tannic acid and selenomethionine treatments during the first 3 months of life reduced the incidence of large hepatocellular carcinoma nodules (diameter >1 cm) in 16-month-old Mdr2-KO mice. We also show here that both chemopreventive agents had an inhibitory effect on gene expression in the livers of Mdr2-KO mice, and that the degree of this inhibition correlated with their ability to decrease portal inflammation at an early age and the incidence of large tumor nodules at a late age: all of these inhibitory activities were higher in the case of selenomethionine treatment.

Materials and Methods

Animal Experiments

Founders of the FVB.129P2-Abcb4^{tm1Bor} (Mdr2-KO; old name, FVB.129P2-P*gy*24^{tm1Bor}) and the wild-type FVB/NJ mice were purchased from the Jackson Laboratory (Bar Harbor, MA). Colonies of both strains were maintained under specific pathogen-free conditions in the Animal Facility of the Hebrew University Medical School. The F1 hybrids produced by breeding of an FVB.129P2-Abcb4^{tm1Bor} male and an FVB/NJ female were used as age-matched controls. Mdr2-KO and Mdr2 heterozygous control mice were divided into three groups: one group received the control diet, and the other two groups received in addition either tannic acid, or seleno-DL-methionine. Tannic acid (Sigma-Aldrich, St. Louis, MO; 300 mg/L) or selenomethionine (Sigma-Aldrich; 8 mg/L) were supplemented in drinking water to pregnant females during last 2 weeks of pregnancy and during weaning and to their progeny until age of 3 months. The average weight of male mice at 3 months of age was ~30 g. Assuming average consumption of drinking water of 5 mL, the daily dose of tannic acid was 1.5 mg (50 mg/kg of animal weight), and the daily dose of selenomethionine was 40 μg (1.33 mg/kg of animal weight). At the age of 3 months, liver tissue samples from three to four males in each group were snap frozen for RNA purification or fixed in formalin for histologic analysis. The remaining mice were maintained on a regular diet until 16 months of age, at which time they were sacrificed for evaluation of liver tumor development. The number of mice sacrificed in each group is outlined in Table 1. Harvesting of liver tissues, RNA purification, and serum biochemistry were done as described previously (7).

Fabrication of cDNA Macroarrays

cDNA probes for macroarray genes were designed using the MacroPrime software, which was created by us specifically for this task. MacroPrime enables developing of PCR-amplified 3′ adjacent cDNA probes for gene expression macroarrays by selecting unique gene regions with appropriate complexity and designing PCR primer pairs for cDNA probe amplification. The software is written in Perl v5.6.1 and Java Platform 1.2; it contains compiled C code of NSEG (22) freely accessible program and exploits the National Center for Biotechnology Information GeneBank, UniGene, and HomoloGene web tools (23) and the Internet version of Primer3 program (24). PCR fragments were obtained by reverse transcription-PCR of total RNA from different mouse tissues and cell lines. The specific DNA bands were extracted from gel and re-amplified by PCR. Reverse transcription-PCR was done by M-MLV reverse transcription-PCR (Promega, Madison, WI) by Super-Therm polymerase (JMR Holdings, London, United Kingdom). Before printing on membranes, all PCR fragments were tested on a 2% agarose gel for product purity and proper size. The cDNA probes were transferred on GeneScreen Plus membranes (NEN, Boston, MA) by BioGrid arrayer (Biorobotics, Cambridge, United Kingdom)

Table 1. Description of mice sacrificed at different stages of the experiment

	No treatment		Tannic acid		Selenomethionine	
	3 mo	16 mo	3 mo	16 mo	3 mo	16 mo
Mdr2-KO	4 males	13 males 8 females	4 males	8 males 17 females	3 males	8 males 12 females
Mdr2$^{+/-}$	4 males	3 males 4 females	3 males	3 males 6 females	4 males	8 males 6 females

in alkaline buffer (0.4 N NaOH). After neutralization, the printed cDNA probes were additionally fixed to membranes by UV irradiation (Fluo-Link, Vilber Lourmat, Paris, France). The macroarrays were stored at −20°C before use.

Labeling and Hybridization with cDNA Macroarrays

The liver RNA samples of three males from each experimental group were subjected to gene expression analysis. Ten micrograms of total RNA were used as a template for reverse transcription by Superscript II enzyme (Invitrogen Life Technologies, Carlsbad, CA) with anchored oligo(dT) primers (25). After RNA denaturation and primer annealing (10 min at 70°C and then 10 min at 4°C) reaction mix containing Superscript II enzyme with its buffer, RNasin (1.6 μg/μL; Promega), 10 mmol/L DTT, 0.5 mmol/L dATP, dGTP, dTTP, and (α-^{33}P)dCTP (Amersham Pharmacia, GE Healthcare, Munich, Germany) was added, and reaction was continued at 42°C during 1 h. Then 0.5 mmol/L dCTP was added, and incubation was continued at 42°C during 30 min. Following heat inactivation of reverse transcriptase (70°C, 15 min), the rest of the RNA was hydrolyzed by 0.25 N NaOH. After neutralization with 0.25 N HCl in 50 mmol/L sodium phosphate buffer, the labeled cDNA probe was separated from deoxynucleotides using mini quick spin DNA columns (Roche Diagnostics, Indianapolis, IN). Pre-hybridization of macroarrays was done in the Church buffer (0.5 mol/L sodium phosphate, 7% SDS, 1 mmol/L EDTA) containing 0.1 mg/mL salmon sperm DNA at 65°C for 1.5 h. Hybridization was done in the Church buffer (0.5 mol/L sodium phosphate buffer, 7% SDS, 1 mmol/L EDTA) containing a labeled probe ($4–7 \times 10^7$ cpm) at 65°C for 24 h. Washing procedure included three steps: 1× SSC, 0.2× SSC, and 0.1× SSC in 0.5% SDS, 4 mmol/L sodium phosphate buffer (pH 7.2) at 65°C (two last washing steps were done twice). All procedures were done with mild rotation in hybridization oven (Heraeus Instruments GmbH, Hanau, Germany).

Signal Visualization and Extraction of the Gene Expression Values

Macroarrays were exposed to a phosphorimager plate for 18 to 24 h and detected with a phosphorimager (BAS-1000, Fuji, Tokyo, Japan). The signal intensities were extracted using the free software VisualGrid (GPC Biotech AG, Martinsried/Munich, Germany) and transformed to raw expression values using the self-made complementary software MembraneProcess (implemented as a Matlab script). The raw expression values were normalized to the intensity of the genome DNA frame. The absent values were replaced with minimal expression value for the corresponding membrane. For each gene, its raw expression value on every membrane was normalized on average expression of this gene in all membranes. For each hybridization experiment, the average of these normalized expression values in two hybridizations was assigned as gene expression value in the specific experiment.

Gene Expression Analysis: Evaluation of the "Therapeutic" Effect of the Treatments

The ''therapeutic'' effect on gene expression profile was defined as an ability of the treatments to reverse the expression pattern of differentially expressed genes in Mdr2-KO mutants. The relation of difference in the expression levels between the treated mutants and untreated heterozygotes to the difference between untreated mutants and heterozygotes had been referred to as a ''reversion index'' of the gene expression pattern and calculated according to the formula: reversion index = $[(X - H) / (M - H)] \times 10$, where X is the average expression value in the treated Mdr2-KO mutants, H is the average expression value in untreated heterozygotes, and M is the average expression value in untreated Mdr2-KO mutants. For reference, when X is equal to H, the reversion index is equal to 0 (as for untreated heterozygotes), whereas when X is equal to M, the reversion index is equal to 10 (as for untreated mutants).

The distance matrix of samples in the space of the differentially expressed genes (2-fold difference in average expression values between the group of untreated Mdr2-KO mutants and the group of untreated Mdr2-heterozygotes with the $P < 0.05$) was calculated and ordered using the SPIN application (26), which arranges the expression profiles in the order of similarity (''side-to-side'' sorting variant).

To study differential expression beyond the single-gene level, we applied a pairwise approach to gene expression analysis (27). For each pair of genes, we calculated its relative expression values for all samples as a difference

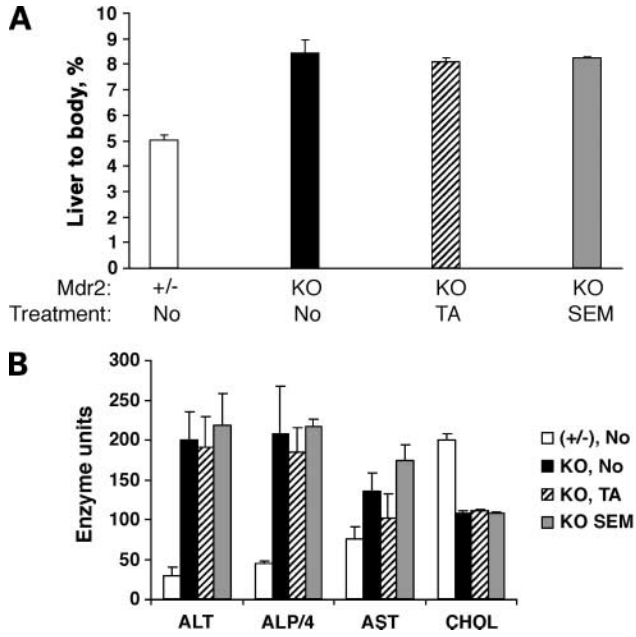

Figure 1. Effect of chemopreventive agents on liver function in 3-month-old mice. **A,** liver to body weight (%). *White,* untreated Mdr2 heterozygotes; *black,* untreated Mdr2-KO; *diagonal hatching,* Mdr2-KO treated with tannic acid; *gray,* Mdr2-KO treated with selenomethionine (four males in each group). **B,** levels of liver enzymes and cholesterol in serum (units). Abbreviations: TA, tannic acid; SEM, selenomethionine; ALT, alanine aminotransferase; ALP/4, alkaline phosphatase level divided into 4 (to fit to the scale); AST, aspartate aminotransferase; CHOL, cholesterol.

between the log 2 transformed expression values of the two genes for each sample and did a *t* test, comparing the relative expression between the pair in the Mdr2-KO mutants and that of the control heterozygotes. There were 134 gene pairs with $P < 0.01$ (*t* test for relative differential expression), and simultaneously, this *P* was less than the *P* obtained by comparing expression levels individually for each of the pair members. Hence, such a pair separates the two groups of samples first, significantly and second, better (as a pair) than each of the two pair members. We refer to such a gene pair as a ''significant gene pair.'' Each sample was now represented by the relative expression levels of the significant gene pairs, and the corresponding sample-to-sample distance matrix was calculated and ordered using the SPIN application (26).

Immunohistochemistry

Formalin-fixed, paraffin-embedded mouse liver tissues were used for immunohistochemistry. Immunostaining with anti–proliferating cell nuclear antigen (anti-PCNA) antibody (sc-56, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was done as described previously (7). Immunostaining with anti–cyclin D1 antibody (Diagnostic BioSystems Inc., Pleasanton, CA) was done in 100 mmol/L glycine buffer (pH 9) by antigen retrieval in a microwave.

Nuclear DNA Fragmentation Assay

Nuclear DNA fragmentation on slides of formalin-fixed, paraffin-embedded mouse liver tissues was detected and quantified using the ''DeadEnd Fluorometric TUNEL system'' (Promega) by an investigator blinded for type of samples (N.K.).

Statistical Analysis

The effects of treatments on tumor incidence and on serum concentration of liver enzymes as well as significance of differential expression of genes were evaluated with the one-tailed *t* test.

Results

Effect of Treatments on Liver Histology, Serum Biochemistry, and Tumor Incidence

To explore the role of oxidative stress and inflammation in hepatocarcinogenesis, we tested the effects of supplementing Mdr2-KO mice with tannic acid and selenomethionine in the early stages of the disease on hepatocellular carcinoma development at advanced stages. We searched for a correlation between liver tumor incidence at the later age and histologic, morphologic, and biochemical variables of treated and untreated mice at an early age. Mdr2-KO and age-matched Mdr2 heterozygous control mice were divided into three groups: one group received the control diet, and the other two groups received in addition either tannic acid, or selenomethionine during the first 3 months of life. At the age of 3 months, liver tissue samples from several males in each group were collected for histologic analysis and RNA purification. Treatment was ceased, and the remaining mice were maintained on a regular diet until 16 months of age, at which time they were sacrificed for evaluation of liver tumor development (Table 1).

The typical pathologic features seen in the liver of Mdr2-KO mice at the age of 3 months are ductular proliferation;

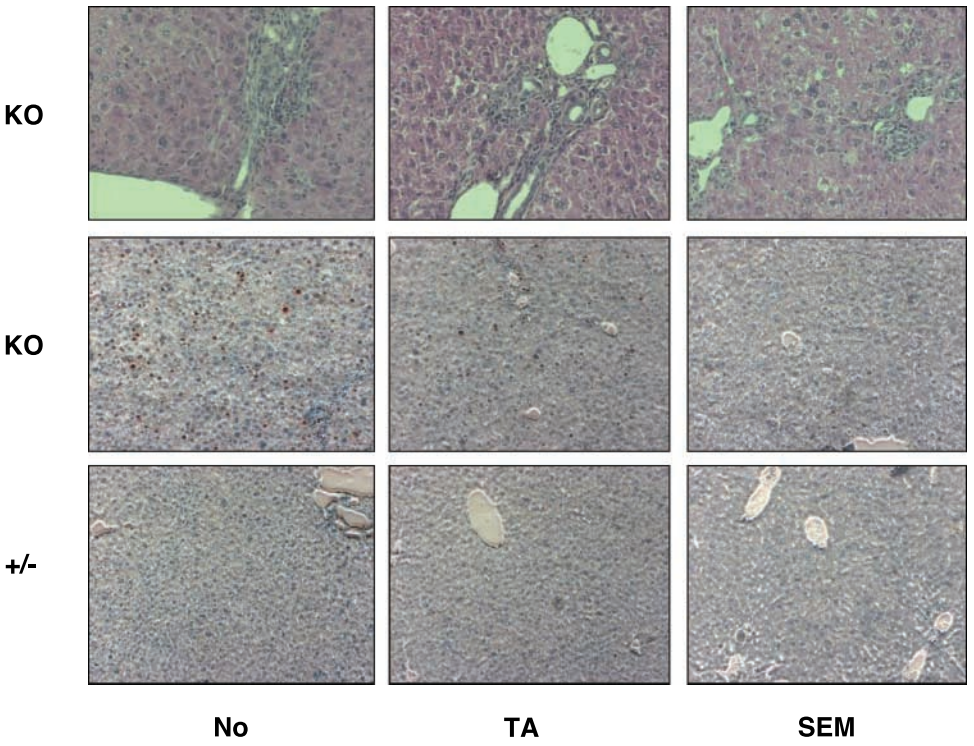

Figure 2. Effect of chemopreventive agents on the liver histology and PCNA expression in 3-mo-old mice. *Top,* H&E staining; *middle* and *bottom,* staining with anti-PCNA antibody. +/−, control Mdr2 heterozygous mice. Abbreviations: KO, Mdr2-KO mice; No, no treatment; TA, treatment with tannic acid; SEM, treatment with selenomethionine.

Table 2. Pathologic scores of liver tissues from 3-mo-old Mdr2-KO and Mdr2$^{+/-}$ control mice either untreated or treated with tannic acid or selenomethionine

Treatment/genotype	Ductular proliferation	Portal inflammation	Fibrosis	Mitotic activity	Councilman bodies	Total score
Untreated						
Mdr2$^{+/-}$	0 ± 0	0 ± 0	0 ± 0	0.5 ± 0.4	0.3 ± 0.5	0.8 ± 0.5
Mdr2-KO	3.0 ± 0	2.7 ± 0.6	2.3 ± 0.6	0.7 ± 0.5	2.0 ± 1.1	10.7 ± 1.5
Tannic acid treated						
Mdr2$^{+/-}$	0 ± 0	0 ± 0	0 ± 0	1.0 ± 1.7	0 ± 0	1.0 ± 1.7
Mdr2-KO	3.0 ± 0	3.0 ± 0	3.0 ± 0	1.0 ± 1.7	0.7 ± 0.6	10.7 ± 2.1
Selenomethionine treated						
Mdr2$^{+/-}$	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.7 ± 0.6	0.7 ± 0.6
Mdr2-KO	2.7 ± 0.6	2.7 ± 0.6	1.3 ± 0.6	0.3 ± 0.6	1.3 ± 0.6	8.3 ± 1.2

NOTE: Pathologic scores were calculated as described in van Nieuwerk et al. (49).

portal inflammation characterized by intense inflammatory cell infiltration and development of varying degrees of fibrosis; hepatomegaly, elevated serum levels of liver enzymes; and reduced levels of serum cholesterol (8). Neither tannic acid nor selenomethionine reduced hepatomegaly, nor changed levels of liver enzymes and cholesterol in the blood (Fig. 1). However, both treatments reduced ductular proliferation and portal inflammation (Fig. 2, *top row*). Histologic evaluation of H&E-stained liver tissues from untreated and treated mice revealed a reduction in levels of fibrosis and some improvement of the total pathologic score in selenomethionine-treated mice, relative to untreated or tannic acid-treated mice (Table 2). To check the effect of selenomethionine on hepatocyte nuclear DNA fragmentation, terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) assay was applied to liver tissue samples of 3-month-old mice sacrificed upon cessation of treatment. Control heterozygotes treated with selenomethionine had very low average number of TUNEL-positive hepatocytes, similar to those of untreated heterozygotes and Mdr2-KO mice. Selenomethionine-treated Mdr2-KO mice had higher average number of TUNEL-positive hepatocytes; however, the difference between treated and untreated Mdr2-KO animals was not statistically significant (data not shown). We have recently shown that PCNA is significantly up-regulated both at RNA and protein levels in the livers of 3-month-old Mdr2-KO mice (7). Here, we show that both treatments reduced PCNA expression, with selenomethionine having a stronger inhibitory effect than tannic acid (Fig. 2, *middle row*). In Mdr2-KO mice sacrificed at the age of 16 months, the total number of tumor nodules and the liver-to-body weight index were similar between untreated and treated groups (data not shown). However, the incidence of large tumors (proportion of mice having tumors with diameter >1 cm) was reduced with tannic acid and selenomethionine treatments (Fig. 3A compared with B); for selenomethionine, this reduction was statistically significant. Of note, neither treatment caused incidence of hyperplasia in the Mdr2$^{+/-}$ control group at 16 months of age.

Analysis of Gene Expression Profiles

To explore the molecular mechanisms of the effects of tannic acid and selenomethionine treatments on Mdr2-KO mice at early stages of the disease, we did gene expression profiling of liver tissue samples taken at the end of treatment period (3 months of age) with our cDNA macroarrays. These arrays were designed and produced in our laboratory. They contained probes to about 250 selected genes that regulate responses to oxidative stress (77 probes) and inflammation (42 probes) as well as genes regulating lipid metabolism (70 probes). Probes to liver-specific (26) and housekeeping (16) genes were included as controls. To design unique gene probes for the macroarray, we developed the MacroPrime software that enables batch gene input, recognizes different gene name standards (and performs orthologic conversion if needed), selects unique gene regions (avoiding low complexity regions), and designs PCR primer pairs for probe amplification. For each experimental group (Mdr2-KO and Mdr2 heterozygotes, untreated, or tannic acid treated, or selenomethionine treated), liver RNA samples from three males were each hybridized with two macroarray membranes. Expression of about 160 genes was detected in a typical hybridization experiment; overall, expression of 178 genes could be compared between untreated and treated Mdr2-KO mice. Comparison of differential expression data between untreated Mdr2-KO and control Mdr2$^{+/-}$ mice, obtained from macroarrays and Affymetrix arrays (7), showed about 70% concordance between two data sets. As it was previously shown (7), many genes that control inflammatory response, lipid metabolism, and response to oxidative stress were differentially expressed (mostly up-regulated) in the livers of untreated 3-month-old Mdr2-KO mice (Supplementary Table S1).[4]

Effects of Chemopreventive Treatments on Gene Expression Profiles

Both tannic acid and selenomethionine treatments significantly inhibited gene expression mainly in the livers of

[4] Supplementary materials for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Mdr2-KO mice and, to a much lesser degree, in control Mdr2 heterozygotes. This inhibition affected genes that either were, or were not, differentially expressed in mutants compared with controls. The inhibitory effect was more prominent in selenomethionine-treated Mdr2-KO mice: the number of genes suppressed by treatments by at least 2-fold (comparing treated versus untreated Mdr2-KO mice) was 23 for tannic acid and 31 for selenomethionine treatment; for changes of 1.8-fold threshold, these numbers were 26 and 46, respectively (Supplementary Table S2).[4] Both treatments also resulted in partial reversion of the expression pattern of genes differentially expressed in Mdr2-KO mice to that of control mice (Supplementary Table S1).[4] Three different approaches were used to estimate this therapeutic effect of the treatments (Fig. 4). For each gene shown in Supplementary Table S1,[4] a ''reversion index'' can be calculated as an average expression level in treated Mdr2-KO mutants relative to its expression levels in untreated mutants (assigned as 10 in an artificial scale) and in control heterozygotes (assigned as 0 in an artificial scale; see Materials and Methods). Figure 4A graphically represents the ''reversion indices'' of the 43 genes that were differentially expressed (at least 2-fold) in untreated Mdr2-KO mutants. The genes are presented in decreasing order of the average reversion index for tannic acid and selenomethionine. The total therapeutic effect of each treatment was calculated as a mean of the ''reversion indices'' of these 43 differentially expressed genes and found to be 5.4 for tannic acid and 5.8 for selenomethionine. Figure 4B represents the distance matrix of samples in the space of differentially expressed genes. It shows that Mdr2-KO mutants and heterozygotes are the maximally remoted groups characterized by a high intrinsic similarity within each group, whereas the treated Mdr2-KO mutants occupy the intermediate positions and are closer to both untreated groups.

Similar results were obtained by pairwise analysis of gene expression data. The main objective in analysis of disease-related gene expression profiles is usually discovering co-regulated gene groups. However, the relations between expression patterns (both direct and reverse) of individual genes also may be of great importance. The genes in our macroarray were functionally related, increasing the chance for discovering regulatory related gene pairs by the pairwise analysis of the expression data. An important feature of such analysis is the robustness of normalization methods due to comparison of the genes from the same membranes. The distance matrix for analyzed samples obtained by the SPIN sorting of relative expression data for gene pairs (see Materials and Methods) is represented on Fig. 4C. It is similar to that of the matrix in Fig. 4B in terms of a maximal distance between intrinsically homogenous groups of untreated Mdr2-KO mutants and heterozygotes and in terms of the intermediate position of treated Mdr2-KO mutants between these two groups. Thus, all three methods represented in Fig. 4 show that both tannic acid and selenomethionine treatments partially reversed the abnormal expression of most genes that were differentially expressed in Mdr2-KO mutants and present on our macroarray.

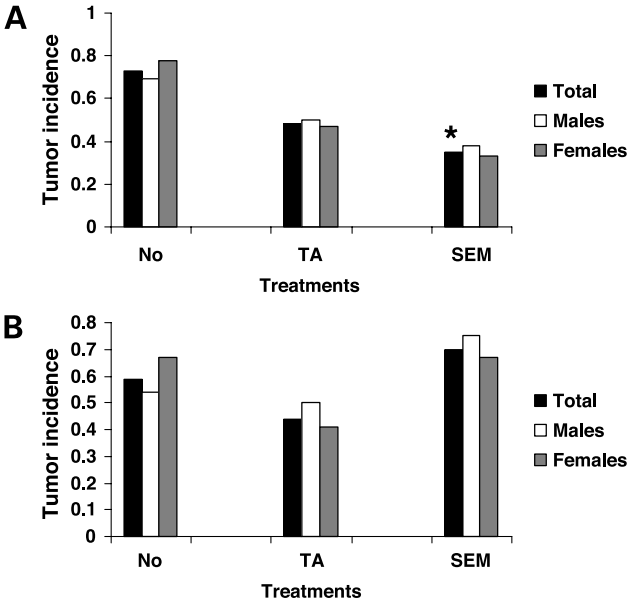

Figure 3. Effect of chemopreventive agents on the incidence of liver tumors in 16-mo-old Mdr2-KO mice. **A,** incidence of tumors with linear size >1 cm. **B,** incidence of tumors with linear size between 0.5 and 1 cm. No, no treatment (13 males and 8 females); TA, treatment with tannic acid (8 males and 17 females); SEM, treatment with selenomethionine (8 males and 12 females). *, $P < 0.05$, statistically significant in comparison with untreated animals by Fisher's exact test.

Discussion

The effect of antioxidant treatment in cancer prevention and therapy is currently a topic of heated debate due to contradicting results emanating from research studies and clinical trials: the outcome of an antioxidant supplement may be beneficial or detrimental, depending upon the patient (28). Oxidative stress has long been implicated in carcinogenesis (29) and shown to correlate with the most important clinical variables of disease (30). It is known that reactive oxygen species activate signal transduction pathways, including activator protein and nuclear factor-κB (NF-κB), and cause oxidative DNA damage, increasing risk of cancer development. However, the exact molecular mechanisms that cause cell transformation and tumor development as well as cell protective mechanisms are not well understood (31, 32). A large amount of data have recently been accumulated showing that different antioxidants act not only as free radical scavengers but also as direct modulators of multiple cell signaling pathways by various mechanisms (33–37).

Chemoprevention against hepatocellular carcinoma in mouse models has previously been achieved with antioxidants, including vitamin E (10), tannic acid (13), and some selenium compounds (11, 12). In most cases, the chemopreventive agent was supplemented during several

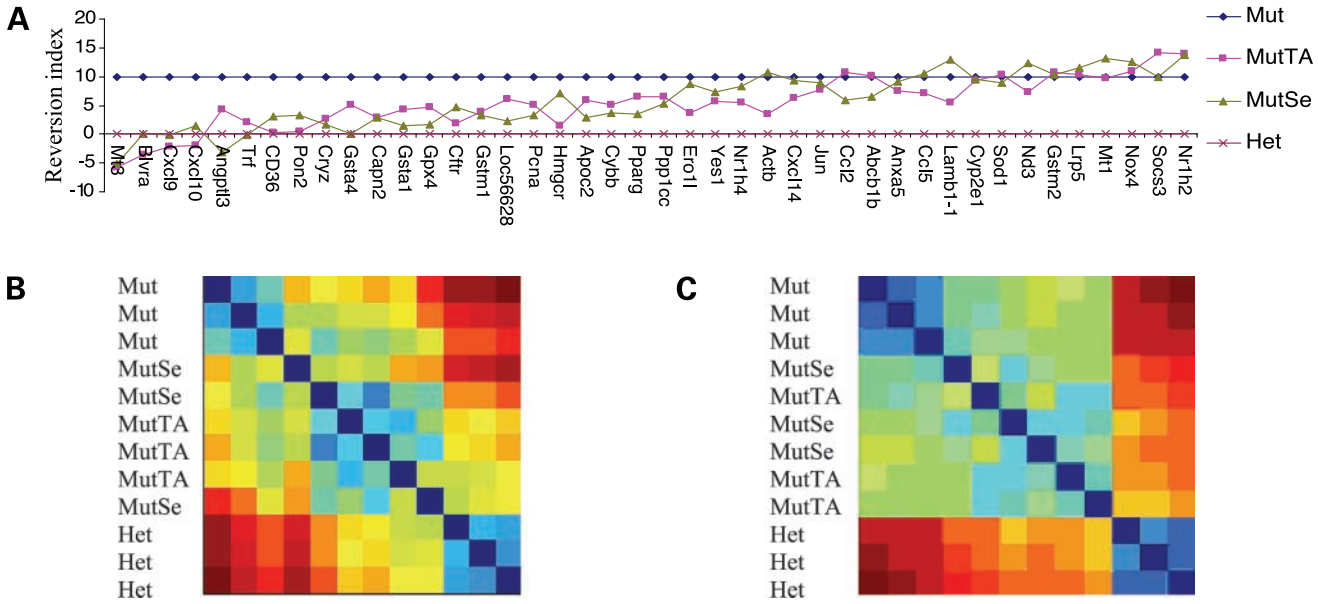

Figure 4. Effect of chemopreventive agents on the expression levels of genes differentially expressed in Mdr2-KO mice. **A,** the reversion indices in a 10-point scale (see Materials and Methods) for untreated Mdr2-KO mutants (*Mut*) and heterozygotes (*Het*) and Mdr2-KO mutants treated with tannic acid (*MutTA*) or selenomethionine (*MutSe*). The indices are sorted in the order of average treatment effect. **B,** distance matrix of samples in the space of differentially expressed genes. The matrix was obtained by data reordering using the SPIN algorithm (see Materials and Methods). The distances are color-coded (the warmer the color, the bigger the distance).The sample order is identical in rows and columns (*diagonal,* distance between each sample and itself). **C,** distance matrix of samples in the space of significant pairs of genes (see Materials and Methods). The only difference with (**B**) is the usage of gene expression values relative for pairs of genes instead of individual values.

months, but in some models, a short prenatal and postnatal exposure resulted in significant decrease of hepatocellular carcinoma incidence (11). Here, we investigated the effect of tannic acid and selenomethionine, supplemented during prenatal period and first 3 months of life, on liver tumor development in the Mdr2-KO mice that represent an inflammation-associated hepatocellular carcinoma model. These mice develop non-suppurative inflammatory cholangitis during the first 3 months, which is characterized by bile acid leakage into portal tracts, increased bile ducts proliferation, and portal inflammation (5, 9). Recently, we have shown induction of antioxidant protection systems and stimulation of hepatocyte DNA replication in the liver of Mdr2-KO mice at the age of 3 months (7). However, hepatocyte mitotic activity has been blocked at this stage. In later stages of the disease, although inflammation was less prominent, and the total antioxidant capacity of liver tissue returned to normal level, mitotic activity of hepatocyte was increased (7). To test the effect of inflammation at early age on liver tumor development at the later ages in this hepatocellular carcinoma model, we treated Mdr2-KO mice with chemopreventive agents during first 3 months of their life, the period of maximal inflammation. Doses were selected based on successful chemoprevention experiments on other mouse cancer models (11, 13, 38).

In contrast to observations in other hepatocellular carcinoma models, our results showed that the applied treatments neither abolished nor significantly reduced overall hepatocellular carcinoma incidence in Mdr2-KO mice. However, the reduced incidence of large tumors may be interpreted as retardation in the initial stages of tumor development. Molecular mechanisms underlying the chemopreventive effect of these two compounds have some common and unique motives. Both compounds down-regulated expression of genes involved in immune/inflammatory and antioxidant responses. However, although on one hand, tannic acid more efficiently inhibited expression of immune/inflammatory genes (*Ccl4, Ccl5, Cd36, Cxcl9*, and *Cxcl10*), selenomethionine more efficiently inhibited expression of antioxidant genes (*Gpx4, Gsta1, Gsta4, Mt1, Prdx3*, and *Prdx6*) on the other.

Tannic acid has anti-inflammatory activities (16); particularly, it was shown that tannic acid protects mice from the tumor necrosis factor-α–induced lethal hepatitis (39). It is also known that some phenolic antioxidants inhibit the NF-κB signaling by blocking the formation of NF-κB-DNA binding complexes (40). We have recently shown that both tumor necrosis factor-α and NF-κB are elevated in inflamed portal tracts of Mdr2-KO mice, and that reduction of tumor necrosis factor-α levels or knocking out NF-κB prevented hepatocellular carcinoma development, providing a rational link between inflammation and tumorigenesis in this model (6). Thus, the chemopreventive effect of tannic acid in Mdr2-KO mice can be attributed, at least in part, to its anti-inflammatory action.

Selenium has proapoptotic (41) and antiangiogenic (42) properties that may be important for cancer chemoprevention. Selenium modulates p53 activity by a redox mechanism

through the redox factor Ref1 (43), or by a phosphorylation mechanism (44), and inhibits NF-κB and p38 signaling pathways (45, 46). On the other hand, it was recently shown that selenium deficiency caused cell death due to accumulation of reactive oxygen species, especially lipid peroxides (47). This effect probably explains the results of Novoselov et al. showing antihepatocarcinogenic effect in a transgenic mouse model both at high levels of selenium and at selenium deficiency but not at intermediate selenium levels (12). Interestingly, most hepatocellular carcinoma cell lines tolerate selenium deficiency probably as a result of selection for survival in oxidative stress–induced selenium-deficient conditions (48). We should point out that the dose of selenomethionine used in our study was not cytotoxic because control selenomethionine-treated heterozygotes had normal levels of liver enzymes in serum, normal liver histology, and normal level of hepatocyte nuclear DNA fragmentation.

We have recently shown that in the liver of Mdr2-KO mice at 3 months of age, PCNA and cyclin D1 expression was highly increased, both at mRNA and protein levels (7). Transcripts of many genes involved in DNA replication and cell cycle regulation were overexpressed, but hepatocyte mitosis was blocked. Such induction of DNA synthesis without subsequent cell division should result in appearance of polyploid hepatocytes, a potential source of chromosomal aberrations. In the majority of tested animal and cell culture cancer models, selenium compounds decreased expression of several cell cycle genes, including *PCNA* and *cyclin D1* (21). In Mdr2-KO mice, selenomethionine inhibited PCNA expression both at the mRNA and protein levels; inhibitory effect of tannic acid on PCNA expression was less pronounced (Fig. 2). Decrease of PCNA levels by selenomethionine may reflect reduction of a signaling that induced excessive hepatocyte DNA replication in Mdr2-KO mice. Nevertheless, cyclin D1 immunohistochemistry showed similarly high nuclear cyclin D1 levels in untreated and tannic acid– or selenomethionine-treated Mdr2-KO mice at the end of treatment period (data not shown). Thus, mechanisms regulating cyclin D1 up-regulation in hepatocytes of Mdr2-KO mice may differ from those in other cancer types. Decrease of the expression of multiple antioxidant genes by selenomethionine (and, to a lesser degree, by tannic acid) may also have beneficial effect on chronic liver disease in Mdr2-KO mice (Table 2). When the antioxidant control mechanisms are exhausted or overrun, the cellular redox potential shifts toward an oxidative stress, increasing the potential for cellular oxidative damage (32).

In conclusion, we showed that supplementing Mdr2-KO mice with tannic acid or selenomethionine during the first 3 months of life, a period of most pronounced inflammation in their livers, had only partial chemopreventive effect: reduction of incidence of large liver tumors at the age of 16 months. This chemopreventive effect was more pronounced (and statistically significant) in the case of selenomethionine treatment. Both treatments had wide inhibitory effect on liver gene expression in Mdr2-KO mice; particularly, they completely or partially reversed expression of many genes that were overexpressed in mutants. The highest chemopreventive activity of selenomethionine correlated with its stronger inhibitory effect of liver gene expression in Mdr2-KO mice, particularly with its stronger down-regulation of PCNA and genes regulating antioxidant protective system. We suggest that chemopreventive activity of selenomethionine can be attributed to its inhibitory effect on expression of multiple genes, including those regulating inflammatory and antioxidant responses, and an excessive DNA replication in hepatocytes of Mdr2-KO mice at early age.

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