Environmental Science & Technology

ARTICLE

pubs.acs.org/est

Genomic Profiling of MicroRNAs and Proteomics Reveals an Early Molecular Alteration Associated with Tumorigenesis Induced by MC-LR in Mice

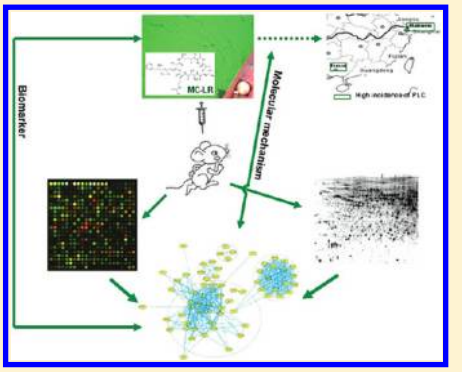
Yanyan Zhao,[†] Ping Xie,[†,*] and Huihui Fan[‡]

[†]Donghu Experimental Station of Lake Ecosystems, State Key Laboratory for Freshwater Ecology and Biotechnology of China, Institute of Hydrobiology, The Chinese Academy of Sciences, Donghu South Road 7, Wuhan 430072, People's Republic of China

[‡]Fisheries College of Huazhong Agricultural University, Wuhan 430070, People's Republic of China

Supporting Information

ABSTRACT: Studies have demonstrated that microcystins (MCs) can act as potential carcinogens and have caused serious risk to public environmental health. The molecular mechanisms of MC-induced susceptibility to carcinogenesis are largely unknown. In this study, we performed for the first time a comprehensive analysis of changes in microRNAs (miRNAs) and proteins expression in livers of mice treated with MC-LR. Utilizing microarray and two-dimensional gel electrophoresis (2-DE) analysis, we identified 37 miRNAs and 42 proteins significantly altered. Many aberrantly expressed miRNAs were related to various cancers (e.g., miR-125b, hepatocellular carcinoma; miR-21, leukemia; miR-16, chronic lymphocytic leukemia; miR-192, pituitary adenomas; miR-199a-3p, ovarian cancer; miR-34a, pancreatic cancer). Several miRNAs (e.g., miR-34a, miR-21) and proteins (e.g., TGM2, NDRG2) that play crucial roles in liver tumorigenesis were first found to be affected by MC-LR in mouse liver. MC-LR also altered the expression of a number of miRNAs and proteins involved in several pathways related to tumorigenesis, such as glutathione metabolism, VEGF signaling, and MAPK signaling pathway. Integration of post-transcriptomics, proteomics, and transcriptomics reveals that the networks miRNAs and their potential target genes and proteins involved in had a close association with carcinogenesis. These results provide an early molecular mechanism for liver tumorigenesis induced by MCs.



INTRODUCTION

Microcystins (MCs) are naturally occurring toxins produced by freshwater cyanobacteria and can be found in lakes, ponds and rivers used for recreational activities as well as sources for drinking water preparation.[1] More than 80 structural analogues of MCs have been identified, with MC-LR being the most toxic and common.[2] MCs primarily act as hepatotoxins, for they are predominantly absorbed, transported and accumulated into liver.[3] Acute exposure to MCs could lead to severe liver damage, including massive intrahepatic hemorrhage, liver swelling, and even animal death.[4] In 1996, there was a tragic incident that led to the death of 60 dialysis patients in Caruaru of Brazil due to the acute exposure to MCs in the water used for dialysis.[5]

There is also evidence that prolonged exposure to MC-LR could induce neoplastic nodular formations that could be precursors of primary liver cancer (PLC).[6] Early studies showed that repeated intraperitoneal (i.p.) injection of MC-LR stimulated the formation of glutathione S-transferase (GST-P) positive foci in rat liver initiated with diethylnitrosamine (DEN).[7,8] These observations indicate that MC-LR is a new liver tumor promoter. Moreover, Ito et al.[9] found that after 100 i.p. injections of a sublethal dose (20 μg/kg) of MC-LR, neoplastic nodules were observed without the use of an initiator, indicating that MC-LR may act as tumor initiators. Epidemiological studies have indicated an association between increased incidence of liver cancer and drinking water sources that were potentially contaminated with MCs in certain areas of China.[10,11] Therefore, chronic exposure to low concentration of MCs through consumption of contaminated water and food may be a serious hazard to human health.

The mechanisms of MC toxicity have not been fully elucidated but most likely involve protein phosphatase (PP1 and PP2A) inhibition leading to disruption of the dynamic equilibrium of protein phosphorylation/dephosphorylation[12] and activation of the expression/activity of downstream transcriptional factors and protein kinases, such as calcium-calmodulin-dependent multifunctional protein kinase II (CaMKII), NIMA (never in mitosis gene a)-related expressed kinase 2 (Nek2), p53, and mitogen-activated protein kinases (MAPKs) which may lead to the

Special Issue: Ecogenomics: Environmental

Received: May 3, 2011
Accepted: September 1, 2011
Revised: August 31, 2011
Published: September 01, 2011

increase of cellular differentiation and proliferation.[13] It is also widely reported that MCs induce the production of reactive oxygen species (ROS) which could cause DNA damage and activate apoptotic pathways.[14]

MicroRNAs (miRNAs) are small noncoding RNA molecules that function as negative regulators of gene expression. In animals, most miRNAs bind to their target mRNAs imperfectly for repressing protein translation at multiple complementary sites within the 3′ untranslated regions (UTRs).[15] MiRNAs play key roles in diverse cellular processes, including development, cell proliferation, apoptosis, response to stress and also function as tumor suppressors or oncogenes.[16,17] Many recent studies suggest that a considerable number of miRNAs are involved in cancer.[16] And most commonly occurring cancers are associated with the aberrant expression of at least one miRNA.[18,19] Moreover, it has been established that specific cancers have their unique miRNA expression profiles,[20] suggesting that miRNAs respond to different cancers in different ways. Thus, miRNA profiles are useful for classifying and identifying cancers. In addition, several studies have been published regarding use of a proteomic-based approach in diagnostic marker identification and signaling pathway elucidation.[21] Protein molecules have direct influences on the development of cancer as it fundamentally arises due to aberrant signaling pathways.[22] Therefore, identifying and understanding the changes of proteins with the related pathways could reveal the dynamics of what is going on in the cell and offer the prognostic categorization of disease condition, as well as in assessing xenobiotics toxicity efficiently.

Although there is currently a consistent knowledge of some of the key processes, such as the increased expression of some proto-oncogenes (e.g., *c-jun*, *c-fos*, *c-myc*) in rat liver exposed to MC-LR,[8,23] the molecular basis of the MCs-induced susceptibility to tumorigenesis is still largely unknown. In the present study, utilizing microarray, two-dimensional electrophoresis (2-DE) and bioinformatics analysis, we for the first time assayed MC-LR-induced changes in the expression of miRNAs and proteins in mouse liver and analyzed the putative pathways and networks that miRNAs and their potential targets involved in combined with the previous transcriptional data. This work would help us fully understand the effects of MC-LR on gene regulations, protein expression and related pathways associated with tumor formation, and provide us a better understanding of the molecular mechanisms of MC-LR induced toxicity and potential carcinogenicity.

MATERIALS AND METHODS

Animal Treatment. 7-week-old virus-free female BALB/c mice were purchased from Wuhan Institute of Virology, CAS. Mice were kept in laboratory animal center and acclimated to the laboratory environment for 7 days before treatment. Twenty mice were randomly assigned to treatment and control groups. Each cage house two or three mice. All cages were located in animal room with temperature ranging from 19 to 21 °C with a 12 h light-dark cycle, and given free access to standard rodent pellet diet and water. All procedures carried out on animals were approved by the Institutional Animal Care and Use Committee, and were in accordance with National Institutes of Health Guide for the Care and Use of laboratory (permit number SCXK 2008−0005).

MC-LR (purity >95%) standards was purchased from Sigma Chemical (St. Louis, MO), dissolved in 0.9% saline solution at desire concentration. Treatment group received i.p. injections of 20 μg MC-LR/kg body weight (bw) per day for 28 days. Control group was injected with the same volume of 0.9% saline solution.

After 28 days of exposure, both the control and MC-LR-treated mice were euthanized by CO2 asphyxiation. The liver tissue was immediately removed, weighed, and stored in liquid nitrogen during necropsy. We then transferred the frozen tissue samples at −80 °C until further analysis.

2-DE and Mass Spectrometry. Proteins were extracted from mouse livers. The first dimension was carried out using 18 cm pH 4−7 IPG gel strips and 350 μL of sample solution. This pH range allowed proteins with similar isoelectric point (pI) values to be separated with higher resolution. After isoelectric focusing (IEF), the gels were subsequently subjected to a second dimensional electrophoresis on 12.5% polyacrylamide gels. In the second dimension, proteins were separated based on their molecular weight. Finally proteins on the 2-DE gels were visualized with Coomassie Brilliant Blue R-250. After gel image analysis, matrix-assisted laser desorption/ionization-time-of-flight (MALDI)-TOF mass specrometry (MS)/MS was used to identify the proteins of interest. Triplicate 2-DE gels were performed for each group (each sample obtained from independent mice from each group). We provide a detailed description of the method in the Supporting Information.

Western Blot Analysis. Liver tissues were lysed in 200 μL of RIPA buffer (0.05 M Tris-HCl [pH 7.4], 0.15 M NaCl, 0.25% deoxycholic acid, 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin). Protein concentrations were measured using the BCA protein assay (Pierce, Rockford, IL). Proteins were separated by SDS-PAGE and transferred to nitroellulose membranes using an electro blotting apparatus (Bio-Rad, America). The blots were probed with the following primary antibodies: Tgm2, Ndrg2 (Santa Cruz Biotechnology, Santa Cruz, CA), and GAPDH (Wuhan Boster Biological Technology Company), followed by incubation in species-matched horseradish peroxidase-conjugated secondary antibodies. The protein signal was developed using NBT/BCIP system. The results of Western blots were quantified with Gene Snap software (Syngene, America).

MiRNA Microarray Assay. Total RNA were extracted from each sample (30−50 mg) using the mirVana miRNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer's instructions. The miRNA microarray analysis was performed by LC Sciences (Houston, TX). Triplicate measurements were performed for each mouse, and there were 3 independent mice samples in each group. We provide a detailed description of the method in the Supporting Information.

MicroRNA Expression Analysis by qPCR. MiRNA expression was quantified using the NCode SYBR GreenER miRNA qRT-PCR kit (Invitrogen) according to the manufacturers' instructions. One microgram of total RNA was used for cDNA synthesis. Reverse transcriptions were carried out in triplicate and analyzed using a Chromo^4 Real-Time Detection System (MJ Research, Cambridge, MA). The relative quantification values for each miRNA were calculated by the $2^{-\Delta\Delta Ct24}$ method using 5S rRNA as an internal reference. The efficiencies of the two primer sets are approximately equal and that they are close to 1.

Bioinformatics and Statistical Analysis. The classification and functions of the proteins identified were obtained by searching Gene Ontology (www.geneontology.org). Predicted mRNA targets were obtained by combining a variety of currently

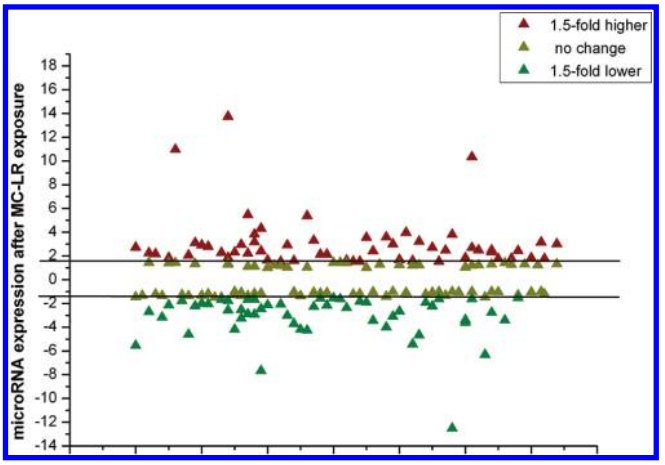


Figure 1. Scatterplot showing miRNAs expression in liver of mice treated with MC-LR. Black lines indicate the 1.5-fold threshold. All values are expressed in fold changes.

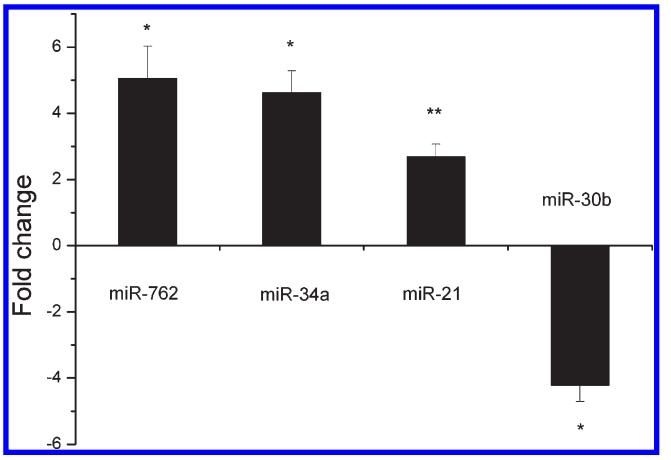


Figure 2. Validation of miRNAs expression. MiR-762, miR-21 and miR-34a were increased in response to MC-LR, whereas miR-30b was decreased in MC-LR-treated samples.

available prediction algorithms miRanda (http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer4.pl), miRBase (http://www.mirbase.org/) and TargetScan (http://www.targetscan.org/mmu_50/). Toxicity pathways were identified by using KEGG PATHWAY (http://www.genome.jp/kegg/pathway.html), PANTHER (http://www.pantherdb.org/genes/) and molecule annotation system (MAS 3.0) (http://bioinfo.capitalbio.com/mas3/).

A two-tailed Student's *t* test was used to determine the significant differences between the control and exposure groups. Statistical analysis was performed using SPSS 13.0 software (SPSS, Chicago, IL), and $p < 0.05$ was considered to be a statistically significant difference.

RESULTS AND DISCUSSION

MC-LR-Induced miRNAs Regulation and the miRNAs Involved in Cancers. The epidemiological surveys for the causes of a high incidence of PLC in Haimen city, Jiangsu province and Fusui country, Guangxi province in China, found a close correlation between the incidence of PLC and the level of MCs in the drinking of pond and ditch water.[10,11] In 2010, the International Agency for Research on Cancer (IARC) classified MC-LR as possible human carcinogen (Group 2B). In the present study, we possibly mark the first time that the effect of MC-LR on the expression of miRNAs which have been demonstrated crucial in carcinogenesis. In this study, 186 miRNAs were identified as being expressed in either control or MC-LR-treated samples (Figure 1). Of the 186 detected miRNAs, 37 were significantly changed ($p < 0.05$). Furthermore, 25 miRNAs induced or repressed ≥3-fold in livers exposed to MC-LR (see Supporting Information, Table S1). To validate the array data, four miRNAs were selected for performing qRT-PCR. Mir-30b was chosen for further analysis because it belongs to miR-30, the only miRNA family with as many as three members decreased by ≥3-fold after MC-LR exposure, while miR-34a, miR-21, and miR-762 were chosen due to their potential crucial roles in liver tumorigenesis induced by MCs. In agreement with the microarray data, each miRNA showed the same trend in regulation, specifically, miR-762, miR-21 and miR-34a each increased in response to MC-LR, whereas miR-30b decreased in MC-LR-treated samples (Figure 2). This miRNA response profile could develop a set of sensitive biomarkers for monitoring and assessing the health effects of MCs and will allow a better understanding of the response spectrum of miRNAs in relation to MCs-induced toxicity and diseases. In this study, many cancer-related miRNAs, such as miR-192, miR-34a, miR-125b, miR-199a-3p, miR-21, and miR-16, were found significantly affected by MC-LR exposure (Table 1). Expression profiles of these miRNAs were significantly altered in many types of cancers, such as breast, lung, ovarian, liver, and prostate cancers, colorectal neoplasia, chronic lymphocytic leukemia, and hepatocellular carcinoma (HCC).[18]

Among these aberrant miRNAs, miR-34a, miR-199−3p and miR-21 have been discovered in HCC and are associated with different etiologies, stage, propensity to recurrence and prognosis as a molecular signature in HCC.[25−27] MiR-34a was the most up-regulated miRNA in response to MC-LR exposure. It is demonstrated that the ectopic expression of miR-34 has rather drastic effects on cell proliferation and survival.[28] As a key trans-acting factor, miR-34a is a necessary mediator in the p53 tumor suppressor activity.[29] The expression of p53 and its downstream genes have been demonstrated to be affected by MCs in many previous studies.[30,31] Several targets of miR-34a including *N-Myc*, *E2f*, *Ccne2*, *Ccnd1*, *Cdk6/4*, *Bcl-2*, *Dll1*, *Sirt1*, *Cdc25c*, *Hmga2*, *Notch1*, *Hdmx*, and *Met* have been discovered, most of which are involved in cell cycle.[32] Some of them (e.g., *Bcl-2*, *Ccnd1*) have been shown to be differentially expressed after MC exposure in previous studies.[33,34] Therefore, this may suggest that MC induces DNA damage leading to the alteration of p53, subsequently altering miR-34a expression and inhibiting the translation of the validated and predicted target genes, and consequently contributing to apoptosis or tumorigenesis in hepatocytes (Figure 3). In addition, previous studies also detected the down-regulation of miR-199a-3p in the majority of HCC samples.[35] Fornari et al.[27] showed contribution of miR-199a-3p to HCC through repressing the translation of *mTOR* and *c-Met*. Furthermore, miR-21 is demonstrated to play an essential role in mediating hepatic oncogenesis and tumor behavior. Meng et al.[25] showed that miR-21 promotes cell invasion, migration, and growth via repression of the phosphatase and tensin homologue (PTEN) expression. Thus, these findings emphasize an essential role of miRNA for liver tumorigenesis, and provide insight into the contribution of altered miRNA expression in contributing to the potential tumor formation induced by MCs.

Table 1. MiRNAs Differentially Expressed in Liver of Mice Exposed to MC-LR and Corresponding Type of Cancers Associated ($p < 0.05$)

MiRNAs with significantly altered expression levels after exposure to MC-LR ($p < 0.05$)	cancers
miR-122 ↓	hepatocellular carcinoma (HCC)
miR-125b ↓	breast cancer, ovarian cancer, HCC
miR-148a ↓	pancreatic cancer
miR-16 ↓	chronic lymphocytic leukemia, pituitary adenomas
miR-192 ↓	pituitary adenomas
miR-199a-3p ↑	ovarian cancer
miR-21 ↑	breast cancer, cholangiocarcinoma, leukemia, cervical cancer
miR-24 ↑	pituitary adenomas
miR-31 ↓	colorectal cancer, lung cancer
miR-34a ↑	pancreatic cancer

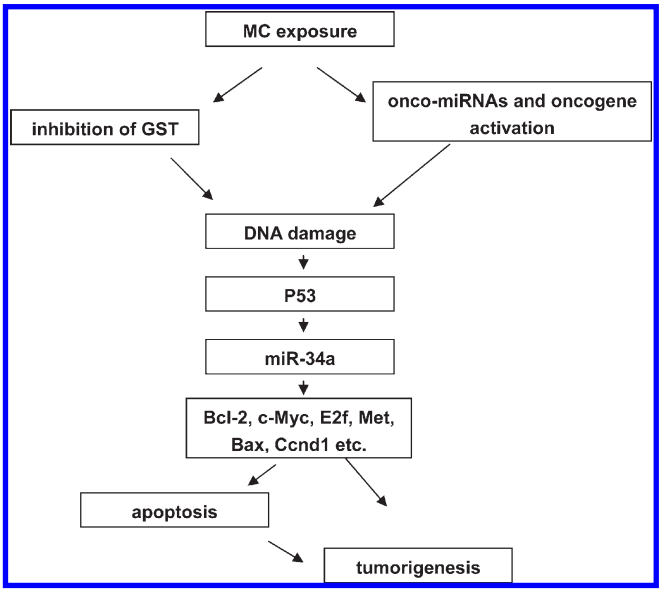


Figure 3. A putative model showing the miR-34a pathway involved in MC-LR induced apoptosis or tumorigenesis. MC-LR modifies the detoxification systems and activates onco-miRNAs and oncogenes leading to DNA damage and p53 alteration, subsequently altering miR-34a expression and inhibiting the translation of the validated and predicted target genes, and consequently inducing apoptosis or tumorigenesis in hepatocytes.

MC-LR-Induced Differentially Expressed Proteins and Their Roles in Cancer. Previous studies showed that exposure to MC-LR induces elevated expression of proto-oncogenes from the *jun*, *fos*, and *myc* gene families which are all involved in the stimulation of cell proliferation.[23,36,37] That is the very early but limited evidence in gene and protein alteration associated with tumorigenesis caused by MC-LR.

In the present study, a 2-fold change cutoff was used as the criterion for differential expression. Compared with the gels from the controls, 62 protein spots were found to have been significantly altered by the effects of MC-LR. A total of 48 protein spots were selected for protein identification based on spot intensity and spot integrity and 42 spots were successfully identified (Figure 4). MC-LR exposure resulted in 17 proteins being up-regulated and 25 being down-regulated (see Supporting Information, Table S2). To validate the result of 2-DE, transglutaminase 2 (TGM2) and N-myc downstream regulated gene 2 (NDRG2) were selected for further Western blot analysis because of their relatively more important roles in liver tumorigenesis. And the expression changes of the selected proteins were consistent with 2-DE results (Figure 5). All the proteins affected by MC-LR were involved in metabolism, hydrolase and transferase activities, regulation of biological process, ion homeostasis, cell communication, binding and response to chemical stimulus. Proteomics now has obvious applications to environmental toxicology as it has the potential both to identify previously unknown protein biomarkers and to gain insights into toxicity mechanisms or related diseases.[38] Among these aberrant proteins, several ones associated with liver tumorigenesis were first found to be affected by MC-LR in mouse liver.

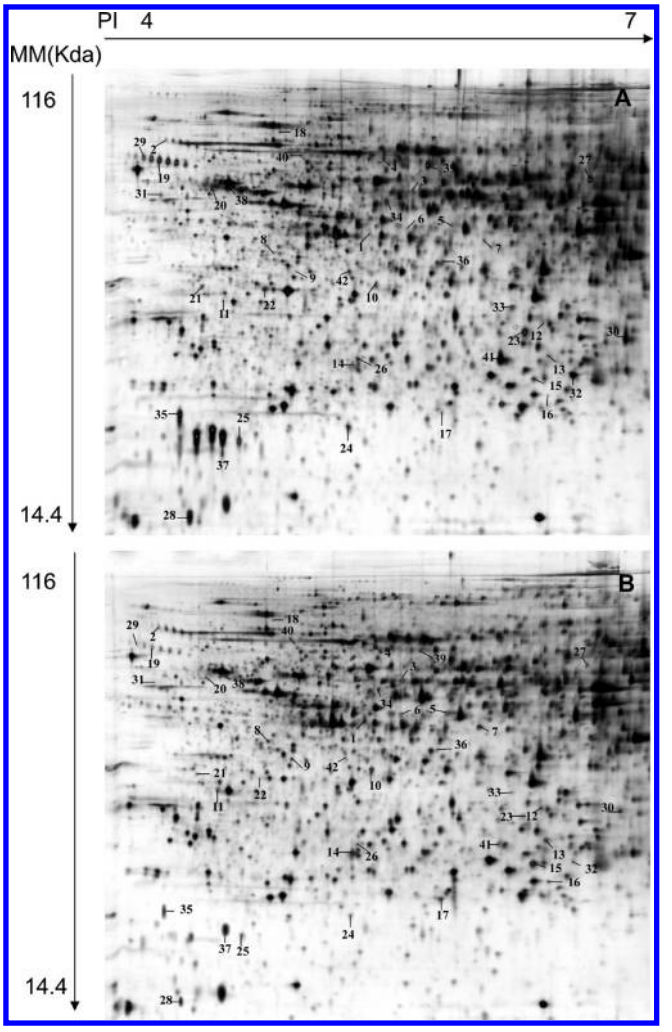


Figure 4. Image of 2-DE gel stained with Coomassie Blue R-250. Mice were treated with MC-LR 20 μg/kg/d for 28 days. Proteins were extracted from liver tissues and then subjected to 2-DE. (A) 2-DE gel image with proteins expressed in the control condition. (B) 2-DE gel image with proteins expressed in the MC-LR-treated condition. Proteins spots that were altered by MC-LR exposure are labeled by characters. The molecular weights (MW) and pI scales are indicated. Each gel is representative of three independent replicates.

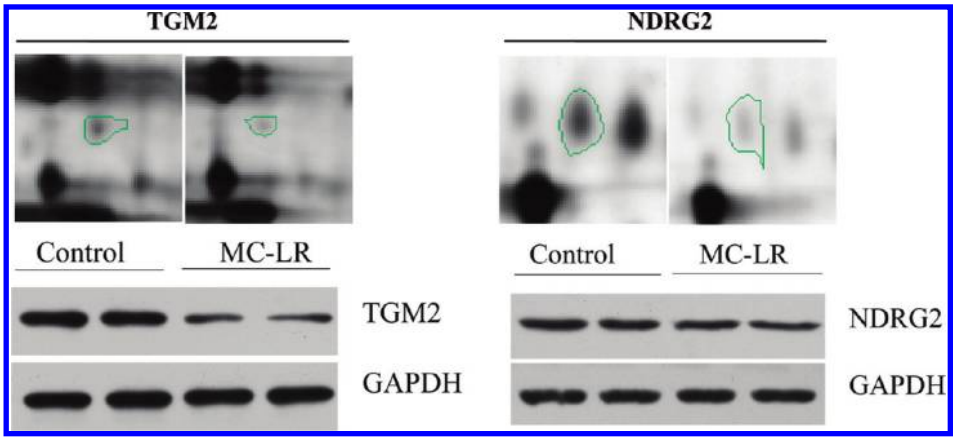


Figure 5. Western blotting analysis. The magnified images of protein spots from the 2-DE gels are shown in the upper part of each panel. The gel pictures presented here were from two independent experiments (n = 3) and showed that protein levels of TGM2 and NDRG2 were significantly reduced in liver of mice exposed to MC-LR.

TGM2 is a ubiquitously expressed protein capable of catalyzing protein/protein cross-linking. TGM2-dependent cross-links are important in extracellular matrix integrity and it has been proposed that this TGM2 activity establishes a barrier to tumor spread.[39] TGM2 was also showed to serve as a well-characterized multifunctional molecule involved in various cellular processes, including differentiation, receptor-mediated endocytosis, apoptosis, cell adhesion, migration[40] and considered playing pivotal role during liver injury and having different functions during hepatocarcinogenesis.[41] In this study, the expression of TGM2 was significantly decreased in liver of mice exposed to MC-LR. Interestingly, Clark et al.[34] detected that mRNA expression of *Tgm2* was increased in liver of mice treated with MC-LR 40 μg/kg/d for 28 days. And in the present study, TGM2 was computationally predicted to be regulated by miR-762 which showed increased expression after MC-LR treatment. Thus, we suggest that the reduction of the final protein level of *Tgm2* might be related to the posttranscriptional regulation of miR-762 after MC-LR exposure. Previous studies have demonstrated that MCs cause apoptosis of hepatocytes through mitochondrial pathway.[4] TGM2 also acts as the upstream regulatory players of the mitochondrial apoptosis pathway.[40] Therefore, the aberrant TGM2 might be a significant biomarker in MC-induced liver injury and is crucial for MC-induced liver tumorigenesis.

NDRG2, a cytoplasmic protein down-regulated by MYC, is involved in cell growth and differentiation, stress, and hormonal responses.[42] NDRG2 was considered as a new tumor suppressor gene candidate.[43] Lee et al.[44] found that the expression of NDRG2 was significantly reduced in HCC and suggested that NDRG2 could suppress tumor cell invasion and migration. Regucalcin (RGN), a novel Ca^{2+}-binding protein, has been shown to play a multifunctional role as a regulatory protein. Endogenous RGN, moreover, has been shown to have an inhibitory effect on DNA and RNA synthesis in the nucleus of normal and regenerating rat liver.[45] Thus, RGN was considered to play a regulatory role in proliferative live cells.[46] In this study, NDRG2 and RGN expressions were both significantly decreased in liver of mice after MC-LR treatment. These two proteins both have critical contributions to liver cell growth and differentiation dependent on their own regular pathways. Thus, the results implicate that these tumor-related proteins could response at the very early stage of liver impairment to MC treatment, and might contribute to MC-induced liver tumorigenesis.

MC-LR Actives Multiple Signaling Pathways in Mouse Liver. MiRNAs are involved in multiple cellular processes through regulating gene and protein expressions. In the present study, we predicted a number of genes to be potential targets of those aberrantly expressed miRNAs through bioinformatic analysis (see Supporting Information, Table S1). Currently, the major strategy to identify miRNA targets is based on various computational programs.[47] In this study, we employed three different algorithms mainly based on seed paring and evolutionary conservation to predict miRNA targets independently and then compared the three lists of target genes predicted by each program. The genes predicted by at least two programs were considered likely to be the real targets of the miRNA. Interestingly, some of these target genes were previously shown affected by MC-LR through microarray analysis in liver of mice treated with 40 μg MC-LR/kg bw/d for 28 days by Clark et al.[34] And this treatment is very close to the condition (MC-LR 20 μg/kg/d for 28 days) of this study. Then, these MC-LR-responsive target genes were divided into four classes by bioinformatic analysis based on both the gene correlations and KEGG pathway analysis, which were glutathione-metabolism-related, glycometabolism-related, ABC-transporters-related, and cancer-related genes (Figure 6). And specially, most genes were involved in cancer related pathways. So this result, to some extent, might explain the MC-LR-induced susceptibility to carcinogenesis of mouse liver and also implicate that the regulation of miRNAs and potential target system might be a significant contributor to these processes after MC-LR exposure.

Furthermore, several differentially expressed proteins were predicted as potential targets of aberrant miRNAs after MC-LR treatment (Table 2). Scientists pointed out that miRNA and their targets system were proved to provide robustness to genetic networks of organism.[48] These potential miRNA-target proteins were involved in 15 pathways and 11 of which were also present in transcript analysis based on the transcriptomic data showed by Clark, et al.[34] These 11 pathways contained glutathione metabolism, metabolism of xenobiotics (e.g., metabolism of xenobiotics by cytochrome P450), glycometabolism (e.g., glycolysis/gluconeogenesis), and several signaling pathways (e.g., MAPK signaling) (Table 3). Glutathione and xenobiotics metabolisms contribute to the detoxification of the MC-LR-induced toxicity in liver. Previous studies have demonstrated that MCs are detoxified in liver through conjugation to glutathione by glutathione

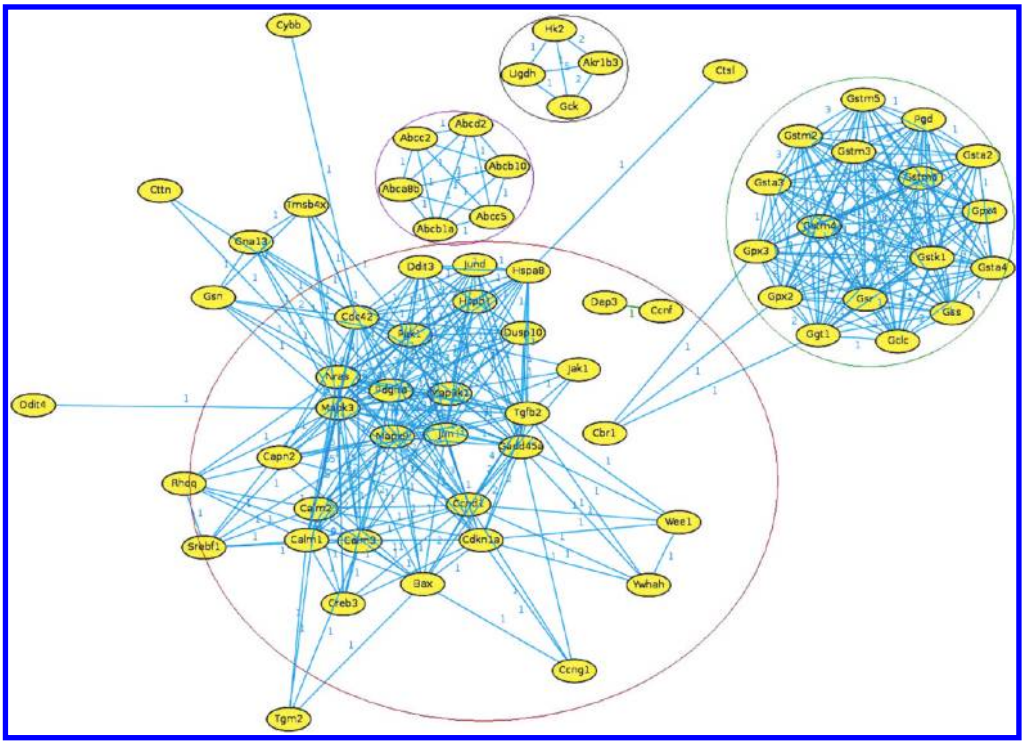


Figure 6. Subnetwork of potential target genes of miRNAs showing an enrichment of genes involved in various pathways. A number of miRNAs potential target genes also previously shown affected by MC-LR were divided into four classes based on both the gene correlations and KEGG pathways analysis. Cancer-related genes (indicated by a red circle); Glutathione-metabolism-related genes (indicated by a green circle); Glycometabolism-related genes (indicated by a black circle); and ABC-transporters-related genes (indicated by a purse circle).

Table 2. Differentially Expressed Proteins That Are Computationally Predicted to Be Regulated by Differentially Expressed miRNAs

miRNA	miRNA fold change	target symbol	protein name	protein fold change
miR-762	3.02	*Tgm2*	transglutaminase 2 (TGM2)	0.33
miR-720	0.29	*Pepd*	Xaa-Pro dipeptidase (PEPD)	2.68
miR-690	0.36	*Nmi*	*N*-myc-interactor (NMI)	2.36
miR-690	0.36	*Pepd*	Xaa-Pro dipeptidase (PEPD)	2.68
miR-455	0.16	*Anxa6*	annexin A6 (ANXA6)	2.64
miR-30c	0.32	*Hspb1*	heat shock protein beta-1 (HSPB1)	3.31
miR-30b	0.25	*Pepd*	Xaa-Pro dipeptidase (PEPD)	2.68
miR-30b	0.25	*Anxa6*	Annexin A6 (ANXA6)	2.64
miR-290−5p	1.54	*Nudt7*	peroxisomal coenzyme A diphosphatase NUDT7 (NUDT7)	0.15
miR-24	3.23	*Mup2*	major urinary protein 2 (MUP2)	0.21
miR-24	3.23	*Mup20*	major urinary protein 20 (MUP20)	0.20
miR-24	3.23	*Mup3*	major urinary protein 3 (MUP3)	0.19
miR-24	3.23	*Fbp1*	fructose-1,6-bisphosphatase 1 (FBP1)	0.48
miR-24	3.23	*Nudt7*	peroxisomal coenzyme A diphosphatase NUDT7 (NUDT7)	0.15
miR-23a	3.97	*Creld2*	cysteine-rich with EGF-like domain protein 2 (CRELD2)	0.32
miR-194	0.23	*Hibadh*	cysteine-rich with EGF-like domain protein 2 (HIBADH)	3.61
miR-192	0.49	*Pepd*	Xaa-Pro dipeptidase (PEPd)	2.68
miR-185	2.93	*Mup20*	major urinary protein 20 (MUP20)	0.20
miR-185	2.93	*Mup3*	major urinary protein 3 (MUP3)	0.19
miR-185	2.93	*Creld2*	cysteine-rich with EGF-like domain protein 2 (CRELD2)	0.32
miR-16	0.41	*Hspb1*	heat shock protein beta-1 (HSPB1)	3.31
miR-16	0.41	*Crym*	mu-crystallin homologue (CRYM)	5.93
miR-148a	0.35	*Pepd*	Xaa-Pro dipeptidase (PEPD)	2.68
miR-125b-5p	0.34	*Pepd*	Xaa-Pro dipeptidase (PEPD)	2.68
miR-101b	0.22	*Crym*	mu-crystallin homologue (CRYM)	5.93

Table 3. List of Pathways Involved in MC-LR Induced Toxicity in Mice Liver[a]

pathways	also present in transcript analysis[b]	MiRNAs	proteins
carbon fixation		miR-24	FBP1
complement and coagulation cascades		miR-455	FGG
cysteine metabolism			CDO1
drug metabolism - cytochrome P450	X	miR-455	GSTP1; GSTM6
fructose and mannose metabolism	X	miR-24	FBP1
glutathione metabolism	X	miR-455	GSTP1; GSTM6
glycolysis/gluconeogenesis	X	miR-24	FBP1
insulin signaling pathway	X	miR-24	FBP1
MAPK signaling pathway	X	miR-16; miR-21; miR-199a-3p; miR-30c; miR-705	HSPB1
metabolism of xenobiotics by cytochrome P450	X	miR-455	GSTP1; GSTM6
pentose phosphate pathway	X	miR-24	FBP1
prostate cancer	X	miR-455	GSTP1
taurine and hypotaurine metabolism	X		CDO1
valine, leucine and isoleucine degradation		miR-290−5p; miR-194	HIBADH
VEGF signaling pathway	X	miR-16; miR-21; miR-199a-3p; miR-30c; miR-705	HSPB1

[a] Each pathway was statistically significant ($p < 0.05$). [b] Pathways analysis based on transcript data from Clark et al.

S-transferase (GST).[49] The alteration of xenobiotics metabolism means that the phase II detoxification systems are modified by MC-LR administration. And the inhibition of detoxification systems plays an important role in genomic damages induced by xenobiotics.[50] DNA damage, a very important factor involved in carcinogenesis, has been widely shown after MCs treatment in previous studies.[33,37] Thus, the MC-LR-induced alteration of glutathione metabolism leading to DNA damages may provide genomic alteration for tumor formation. Therefore, MC-LR-induced changes in expression of genes, proteins, and related miRNAs involved in those pathways have the potential to contribute to MC-induced health effects, especially the tumor promotion, a major concern on public environmental health.

■ ASSOCIATED CONTENT

Supporting Information. Details on the materials and methods, including sample preparation for 2-DE, 2-DE detection, image acquisition and analysis, mass spectrometric analysis, and miRNA microarray measurement; Table S1 showing miRNAs induced or repressed ≥3-fold in mouse livers exposed to MC-LR; Table S2 showing a list of proteins differentially expressed in mouse liver after MC-LR exposure. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone/fax: +86 27 68780622; e-mail: xieping@ihb.ac.cn.

■ ACKNOWLEDGMENT

We greatly appreciate the useful comments and suggestions by Dr. Gary S. Sayler and two anonymous reviewers. The authors would like to thank Dr. Jun Chen in donghu experimental station of lake ecosystems, institute of hydrobiology, China academy of science for her generous help during our research. This study was jointly supported by the National Basic Research Program of China (973 Program) (2008CB418101), the National Natural Science Foundation of China (31070457) and State Key Laboratory of Freshwater Ecology and Biotechnology (2008FBZ01).

■ REFERENCES

(1) Chorus, I.; Falconer, I. R.; Salas, H. J.; Bartram, J. Health risks caused by freshwater cyanobacteria in recreational waters. *J. Toxicol. Environ. Health, Part B* **2000**, *3*, 323–347.

(2) Dietrich, D.; Hoeger, S. Guidance values for microcystins in water and cyanobacterial supplement products (blue-green algal supplements): a reasonable or misguided approach? *Toxicol. Appl. Pharmacol.* **2005**, *203*, 273–289.

(3) Dahlem, A. M.; Hassan, A. S.; Swanson, S. P.; Carmichael, W. W.; Beasley, V. R. A model system for studying the bioavailability of intestinally administered microcystin-LR, a hepatotoxic peptide from the cyanobacterium Microcystis aeruginosa. *Pharmacol. Toxicol.* **1989**, *64*, 177–181.

(4) Ding, W. X.; Shen, H. M.; Ong, C. N. Calpain activation after mitochondrial permeability transition in microcystin-induced cell death in rat hepatocytes. *Biochem. Biophys. Res. Commun.* **2002**, *291*, 321–331.

(5) Pouria, S.; de Andrade, A.; Barbosa, J.; Cavalcanti, R. L.; Barreto, V. T.; Ward, C. J.; Preiser, W.; Poon, G. K.; Neild, G. H.; Codd, G. A. Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. *Lancet* **1998**, *352*, 21–26.

(6) Leong, T. Y.; Leong, A. S. Epidemiology and carcinogenesis of hepatocellular carcinoma. *HPB (Oxford)* **2005**, *7*, 5–15.

(7) Nishiwaki-Matsushima, R.; Ohta, T.; Nishiwaki, S.; Suganuma, M.; Kohyama, K.; Ishikawa, T.; Carmichael, W. W.; Fujiki, H. Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. *J. Cancer Res. Clin. Oncol.* **1992**, *118*, 420–424.

(8) Ohta, T.; Sueoka, E.; Iida, N.; Komori, A.; Suganuma, M.; Nishiwaki, R.; Tatematsu, M.; Kim, S. J.; Carmichael, W. W.; Nodularin, H. F. A potent inhibitor of protein phosphatases 1 and 2A, is a new environmental carcinogen in male F344 rat liver. *Cancer Res.* **1994**, *54*, 6402–6406.

(9) Ito, E.; Kondo, F.; Terao, K.; Harada, K. I. Neoplastic nodular formation in mouse liver induced by repeated intraperitoneal injections of microcystin-LR. *Toxicon* **1997**, *35*, 1453–1457.

(10) Yu, S. Z. Drinking water and primary liver cancer. In *Primary Liver Cancer*; Tang, Z.-Y.; Wu, M.-C.; Xia, S.-S., Eds.; China Academic Publishers: Beijing, 1989; pp 30−37.

(11) Ueno, Y.; Nagata, S.; Tsutsumi, T.; Hasegawa, A.; Watanabe, M. F.; Park, H. D.; Chen, G. C.; Chen, G.; Yu, S. Z. Detection ofmicrocystins, a blue-green algal hepatotoxin, in drinking water sampled

in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay. *Carcinogenesis* **1996**, *17*, 1317–1321.

(12) Fujiki, H.; Suganuma, M. Unique features of the okadaic acid activity class of tumor promoters. *J Cancer Res. Clin. Oncol.* **1999**, *125*, 150–155.

(13) Campos, A.; Vasconcelos, V. Molecular mechanisms of microcystins toxicity in animal cells. *Int. J. Mol. Sci.* **2010**, *11*, 268–287.

(14) Svirčev, Z.; Baltić, V.; Gantar, M.; Juković, M.; Stojanović, D.; Baltić, M. Molecular aspects of microcystins-induced hepatotoxicity and hepatocarcinogenesis. *J. Environ. Sci. Health, Part C: Environ. Carcinog. Ecotoxicol. Rev.* **2010**, *28*, 39–59.

(15) Kwak, P. B.; Iwasaki, S.; Tomari, Y. The microRNA pathway and cancer. *Cancer Sci.* **2010**, *101*, 2309–2315.

(16) Calin, G. A.; Croce, C. M. MicroRNA signatures in human cancers. *Nat. Rev. Cancer* **2006**, *6*, 857–866.

(17) Esquela-Kerscher, A.; Slack, F. J. Oncomirs-microRNAs with a role in cancer. *Nat. Rev. Cancer* **2006**, *6*, 259–269.

(18) Zhang, B. H.; Pan, X. P.; Cobb, G. P.; Anderson, T. A. microRNAs as oncogenes and tumor suppressors. *Dev. Biol.* **2007a**, *302*, 1–12.

(19) Zhang, B. H.; Pan, X. P. RDX induces aberrant expression of microRNAs in mouse brain and liver. *Environ. Health Perspect.* **2009**, *117*, 231–240.

(20) Lu, J.; Getz, G.; Miska, E. A.; Alvarez-Saavedra, E.; Lamb, J.; Peck, D.; Sweet-Cordero, A.; Ebert, B. L.; Mak, R. H.; Ferrando, A. A.; Downing, J. R.; Jacks, T.; Horvitz, H. R.; Golub, T. R. MicroRNA expression profiles classify human cancers. *Nature* **2005**, *435*, 834–838.

(21) Joshi, S.; Tiwari, A. K.; Mondal, B.; Sharma, A. Oncoproteomics. *Clin. Chim. Acta* **2011**, *412*, 217–226.

(22) Joshi, S.; Tiwari, A. K.; Mondal, B.; Sharma, A. Oncoproteomics. *Clin. Chim. Acta* **2011**, *412*, 217–226.

(23) Li, H.; Xie, P.; Li, G.; Hao, L.; Xiong, Q. In vivo study on the effects of microcystin extracts on the expression profiles of proto-oncogenes (c-fos, c-jun and c-myc) in liver, kidney and testis of maleWistar rats injected i.v.with toxins. *Toxicon* **2009**, *53*, 169–175.

(24) Livak, K. J.; Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T) (-Delta Delta C) method. *Methods* **2001**, *25*, 402–408.

(25) Meng, F.; Henson, R.; Wehbe-Janek, H.; Ghoshal, K.; Jacob, S.; Patel, T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology* **2007**, *133*, 647–658.

(26) Cole, K. A.; Attiyeh, E. F.; Mosse, Y. P.; Laquaglia, M. J.; Diskin, S. J.; Brodeur, G. M.; Maris, J. M. A functional screen identifies miR-34a as a candidate neuroblastoma tumor suppressor gene. *Mol. Cancer Res.* **2008**, *6*, 735–742.

(27) Fornari, F.; Milazzo, M.; Chieco, P.; Negrini, M.; Calin, G. A.; Grazi, G. L.; Pollutri, D.; Croce, C. M.; Bolondi, L.; Gramantieri, L. MiR-199a-3p regulates mTOR and c-Met to influence the doxorubicin sensitivity of human hepatocarcinoma cells. *Cancer Res.* **2010**, *70*, 5184–5193.

(28) Tivnan, A.; Tracey, L.; Buckley, P. G.; Alcock, L. C.; Davidoff, A. M.; Stallings, R. L. MicroRNA-34a is a potent tumor suppressor molecule in vivo in neuroblastoma. *BMC Cancer* **2011**, *11*, 33–43.

(29) Raver-Shapira, N.; Marciano, E.; Meiri, E.; Spector, Y.; Rosenfeld, N.; Moskovits, N.; Bentwich, Z.; Oren, M. Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. *Mol. Cell* **2007**, *26*, 731–743.

(30) Takumi, S.; Komatsu, M.; Furukawa, T.; Ikeda, R.; Sumizawa, T.; Akenaga, H.; Maeda, Y.; Aoyama, K.; Arizono, K.; Ando, S.; Takeuchi, T. p53 Plays an important role in cell fate determination after exposure to microcystin-LR. *Environ. Health Perspect.* **2010**, *118*, 1292–1298.

(31) Clark, S. P.; Ryan, T. P.; Searfoss, G. H.; Davis, M. A.; Hooser, S. B. Chronic Microcystin Exposure Induces Hepatocyte Proliferation with Increased Expression of Mitotic and Cyclin-associated Genes in P53-deficient Mice. *Toxicol. Pathol.* **2008**, *36*, 190–203.

(32) Hermeking, H. The miR-34 family in cancer and apoptosis. *Cell Death Differ.* **2010**, *17*, 193–199.

(33) Chen, T.; Wang, Q.; Cui, J.; Yang, W.; Shi, Q.; Hua, Z.; Ji, J.; Shen, P. Induction of apoptosis in mouse liver by microcystin-LR: A combined transcriptomic, proteomic, and simulation strategy. *Mol. Cell. Proteomics* **2005**, *4*, 958–974.

(34) Clark, S. P.; Davis, M. A.; Ryan, T. P.; Searfoss, G. H.; Hooser, S. B. Hepatic gene expression changes in mice associated with prolonged sublethal microcystin exposure. *Toxicol. Pathol.* **2007**, *35*, 594–605.

(35) Gramantieri, L.; Ferracin, M.; Fornari, F.; Veronese, A.; Sabbioni, S.; Liu, C. G.; Calin, G. A.; Giovannini, C.; Ferrazzi, E.; Grazi, G. L.; Croce, C. M.; Bolondi, L.; Nergini, M. Cyclin G1 is a target of miR-122a, a microRNA frequently down-regulated in human hepatocellular carcinoma. *Cancer Res.* **2007**, *67*, 6092–6099.

(36) Sueoka, E.; Sueoka, N.; Okabe, S.; Kozu, T.; Komori, A.; Ohta, T.; Suganuma, M.; Kim, S. J.; Lim, I. K.; Fujiki, H. Expression of the tumor necrosis factor a gene and early response genes by nodularin, a liver tumor promoter, in primary cultured rat hepatocytes. *J. Cancer Res. Clin. Oncol.* **1997**, *123*, 413–419.

(37) Žegura, B.; Zajc, I.; Lah, T. T.; Filipič, M. Patterns of microcystin-LR induced alteration of the expression of genes involved in response to DNA damage and apoptosis. *Toxicon* **2008**, *51*, 615–623.

(38) Dowling, V. A.; Sheehan, D. Proteomics as a route to identification of toxicity targets in environmental toxicology. *Proteomics* **2006**, *6*, 5597–5604.

(39) Fésüs, L.; Piacentini, M. Transglutaminase 2: an enigmatic enzyme with diverse funcions. *Trends Biochem. Sci.* **2002**, *27*, 534–539.

(40) Mehta, K.; Kumar, A.; Kim, H. I. Transglutaminase 2: A multi-tasking protein in the complex circuitry of inflammation and cancer. *Biochem. Pharmacol.* **2010**, *80*, 1921–1929.

(41) Sun, Y.; Mi, W.; Cai, J. Q.; Ying, W. T.; Liu, F.; Lu, H. Z.; Qiao, Y. Y.; Jia, W.; Bi, X. Y.; Lu, N.; Liu, S. M.; Qian, X. H.; Zhao, X. H. Quantitative proteomic signature of liver cancer cells: Tissue transglutaminase 2 could be a novel protein candidate of human hepatocellular carcinoma. *J. Pro. Res.* **2008**, *7*, 3847–3859.

(42) Boulkroun, S.; Fay, M.; Zennaro, M. C.; Escoubet, B.; Jaisser, F.; Blot-Chabaud, M.; Farman, N.; Courtois-Coutry, N. Characterization of rat NDRG2 (N-Myc downstream regulated gene 2), a novel early mineralocorticoid-specific induced gene. *J. Biol. Chem.* **2002**, *277*, 31506–31515.

(43) Liu, N.; Wang, L.; Liu, X.; Yang, Q.; Zhang, J.; Zhang, W.; Wu, Y.; Shen, L.; Zhang, Y.; Yang, A.; Han, H.; Zhang, J.; Yao, L. Promoter methylation, mutation, and genomic deletion are involved in the decreased NDRG2 expression levels in several cancer cell lines. *Biochem. Biophys. Res. Commun.* **2007**, *358*, 164–169.

(44) Lee, D. C.; Kang, Y. K.; Kim, W. H.; Jang, Y. J.; Kim, D. J.; Park, I. Y.; Sohn, B. H.; Sohn, H. A.; Lee, H. G.; Lim, J. S.; Kim, J. W.; Song, E. Y.; Kim, D. M.; Lee, M. N.; Oh, G. T.; Kim, S. J.; Park, K. C.; Yoo, H. S.; Choi, J. Y.; Yeom, Y. I. Functional and clinical evidence for NDRG2 as a candidate suppressor of liver cancer metastasis. *Cancer Res.* **2008**, *68*, 4210–4220.

(45) Yamaguchi, M. Role of regucalcin in maintaining cell homeostasis and function. *Int. J. Mol. Med.* **2005**, *15*, 372–389.

(46) Yamaguchi, M. The transcriptional regulation of regucalcin gene expression. *Mol. Cell. Biochem.* **2011**, *346*, 147–171.

(47) Zhang, B. H.; Pan, X. P.; Wang, Q. L.; Cobb, G. P.; Anderson, T. A. Computational identification of microRNAs and their targets. *Comput. Biol. Chem.* **2006**, *30*, 395–407.

(48) Hornstein, E.; Shomron, N. Canalization of development by microRNAs. *Nat. Genet.* **2006**, *38*, S20–S24.

(49) Takenaka, S. Covalent glutathione conjugation to cyanobacterial hepatotoxin microcystin LR by F344 rat cytosolic andmicrosomal glutathione S-transferases. *Environ. Toxicol. Pharmacol.* **2001**, *9*, 135–139.

(50) Schuliga, M.; Chouchane, S.; Snow, E. T. Upregulation of glutathione-related genes and enzyme activities in cultured human cells by sublethal concentrations of inorganic arsenic. *Toxicol. Sci.* **2002**, *70*, 183–192.