Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Effect of microcystin-LR on protein phosphatase 2A and its function in human amniotic epithelial cells*

Jing LIANG[1], Tan LI[1], Ya-li ZHANG[1], Zong-lou GUO[†‡2], Li-hong XU[†‡1]

([1]*Department of Biochemistry and Genetics, School of Medicine, Zhejiang University, Hangzhou 310058, China*)

([2]*Department of Biosystem Engineering, College of Biosystem Engineering and Food Science, Zhejiang University, Hangzhou 310058, China*)

[†]E-mail: zlguo@zju.edu.cn; xulihong@zju.edu.cn

Received Apr. 13, 2011; Revision accepted July 27, 2011; Crosschecked Nov. 14, 2011

Abstract: Due to their toxicity, the increased distribution of microcystins (MCs) has become an important worldwide problem. MCs have been recognized as inhibitors of protein phosphatase 2A (PP2A) through their binding to the PP2A catalytic subunit. However, the exact mechanism of MC toxicity has not been elucidated, especially concerning the cellular response and its autoregulation. To further dissect the role of PP2A in MC-induced toxicity, the present study was undertaken to determine the response of PP2A in human amniotic epithelial (FL) cells treated with microcystin-LR (MCLR), one of the MC congeners. The results show that a low-dose treatment of MCLR in FL cells for 6 h induced an increase in PP2A activity, and a high-dose treatment of MCLR for 24 h decreased the activity of PP2A, as expected. The increased mRNA and protein levels of the PP2A C subunit may explain the increased activity of PP2A. Furthermore, MCLR altered microtubule post-translational modifications through PP2A. These results further clarify the underlying mechanism how MCLR affects PP2A and may be helpful for elucidating the complex toxicity of MCLR.

Key words: Microcystin-LR, Protein phosphatase 2A, Phosphatase activity, Hormesis, Tubulin, B55α
doi:10.1631/jzus.B1100121 **Document code:** A **CLC number:** Q26

1 Introduction

Cyanobacterial blooms and the adverse effects of their toxins are severe health threats to both animals and humans (Jochimsen *et al.*, 1998; Pinho *et al.*, 2003; dos S. Vieira *et al.*, 2005). Microcystins (MCs) are a family of monocyclic heptapeptide hepatotoxins produced by freshwater species of cyanobacteria, such as *Microcystis aeruginosa*. There are more than 80 kinds of MC congeners, of which microcystin-LR (MCLR) is one of the most toxic (Hoeger *et al.*, 2005). It has been demonstrated that the liver is the prime target organ affected by MCs (Robinson *et al*., 1989). Thus, the hepatotoxicity of MCs has been extensively investigated, both in vivo and in vitro (Ding *et al*., 1998; Malbrouck *et al*., 2003; Fu *et al*., 2005). In addition, the potent tumor-promoting activity of MCs in animals and epidemiological surveys showing the relationship between the MC content of drinking water and human cancer have been of great concern (Yu, 1995; Ito *et al*., 1997; Humpage *et al*., 2000; Hu *et al*., 2002). The induction of apoptosis in a wide variety of cell types and organs is the typical cellular effect of MCs and is closely related to their toxicities (Huang *et al*., 2011; McDermott *et al*., 1998; Ding *et al*., 2000a; Chen *et al*., 2005; Li *et al*., 2009). Oxidative damage also contributes to the harmful effect exerted by MCs on organisms (Ding *et al*., 2000b; Moreno *et al*., 2005; Clark *et al*., 2007). Yoshizawa *et al.* (1990) found that MCs are potent inhibitors of protein phosphatase 1 (PP1) and 2A (PP2A), and this inhibition was responsible for the tumor-promoting activity of MCs. This finding has been considered to be a milestone in MC research. Specifically, PP2A is

‡ Corresponding authors

* Project supported by the National Natural Science Foundation of China (Nos. 30771827 and 20777067) and the Key Special Program on the S&T of China for the Pollution Control and Treatment of Water Bodies (No. 2008ZX07421-001)

© Zhejiang University and Springer-Verlag Berlin Heidelberg 2011

more sensitive to MCs, and it has been confirmed that the half maximal inhibitory concentration (IC_{50}) values of PP1 and PP2A are 1.7 nmol/L and 40 pmol/L, respectively. As PP2A has been recognized to be an important player in many essential aspects of cell function (Janssens and Goris, 2001), pathology due to the inhibition of PP2A by MCs has been an attractive research focus, both for investigating the MC toxicity mechanism and for probing PP2A function.

PP2A holoenzymes are heterotrimers that consist of a structural subunit A, a catalytic subunit C, and a regulatory subunit B (Janssens and Goris, 2001). The B subunit family is thought to mediate substrate specificity and the subcellular localization of the PP2A holoenzyme, and its abnormity is involved in many diseases (Chen *et al.*, 2004; Grochola *et al.*, 2009; Vazquez *et al.*, 2011). For instance, B55α is thought to be related to the acetylation and detyrosination of tubulin, and reduced levels of neuronal B55α-containing PP2A heterotrimers might contribute to microtubule (MT) destabilization in Alzheimer's disease (Nunbhakdi-Craig *et al.*, 2007). Aside from subunit composition, the activity of PP2A is also regulated by post-translational modifications of the C subunit, including phosphorylation, methylation, and nitration. The phosphorylation of Tyr^{307} on the catalytic subunit of PP2A (PP2Ac) can decrease its phosphatase activity (Chen *et al.*, 1992). The methylation of Leu^{309} on PP2Ac critically modulates the binding of regulatory subunits to the AC core enzyme, thereby affecting PP2A substrate specificity, activity, and cellular function (Tolstykh *et al.*, 2000; Xing *et al.*, 2006; Xu *et al.*, 2006). Xing *et al.* (2006) have confirmed that MCLR is covalently bound in a pocket at the active sites of the PP1 and PP2A catalytic subunits. Furthermore, studies in our laboratory have revealed that exposure to MCLR, MCRR, and bloom extract increases the protein levels of the A subunit of PP2A in vivo and in vitro (Huang *et al.*, 2008; Xing *et al.*, 2008; Fu *et al.*, 2009). Therefore, the pattern of MC effects on PP2A is much more complicated than previously thought.

The normal human amnion-derived FL cell line (Fogh and Lund, 1957) has been used in many studies, including our previous works (Liu *et al.*, 2006; Shen *et al.*, 2006; Wu *et al.*, 2006; Zhu *et al.*, 2007), and has proven to be an adequate model to explore the toxic effects of exotic compounds. In addition to aforementioned up-regulation of the PP2A A subunit in FL cells, our studies also indicated that the apoptosis-related proteins Bcl-2, Bax, and p53 are involved in FL cells treated with MCLR (Xing *et al.*, 2008). Therefore, additional work on FL cells treated with MCLR will provide an in-depth study that can reveal the MC toxicity mechanism. The present study was designed to determine the change in PP2A activity in FL cells to confirm the time and dose effects of MCLR on PP2A activity. Furthermore, the phosphorylation of the PP2Ac subunit was studied to obtain a comprehensive understanding of MCLR on PP2A. As an important illustration reflecting the regulatory role of PP2A in MT stabilization, B55α, acetylated tubulin, and detyrosinated tubulin were investigated synchronously to show MCLR's destructive effect on PP2A. These results will further clarify the underlying mechanism of MCLR's effects on PP2A and may be extremely helpful for elucidating the complex toxicity of MCLR.

2 Materials and methods

2.1 Materials

MCLR was from Alexis (Carlsbad, CA). Dimethylsulfoxide (DMSO) and 4′,6-diamidino-2-phenylindole (DAPI) were from Sigma (St. Louis, MO). Powdered minimum essential medium (MEM) was from Gibco (Scotland, UK). Fetal bovine serum (FBS) was purchased from Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). The anti-PP2A C antibody was obtained from Cell Signaling (Danvers, MA). The anti-MCLR antibody was from Alexis. The anti-phospho-PP2Ac (Tyr^{307}), anti-acetylated tubulin, and anti-α-tubulin antibodies were from Abcam (Cambridge, MA). The anti-Tyr tubulin antibody was from Sigma. The anti-β-actin antibody and enhanced chemiluminescence (ECL) detection kit were purchased from Santa Cruz (Santa Cruz, CA). All of the horseradish peroxidase (HRP)-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). All other chemicals were of the highest grade available from commercial sources.

2.2 Cell culture and treatment

The human amniotic epithelial cells (FL) were

from the American Type Culture Collection (CCL-62) and cultured on MEM supplemented with 10% (v/v) heat-inactive FBS and 2.2 g/L $NaHCO_3$ at 37 °C and 5% CO_2 until 70%–80% confluence was reached. DMSO was used as a solvent control, and the concentration was 0.1% (v/v). Cells in the logarithmic growth phase were exposed to various doses of MCLR or DMSO (control). At the designated time point, the cells were washed twice with ice-cold phosphate buffered saline (PBS) before harvest, followed by four gentle rinses with PBS and pelleting by centrifugation each time to fully remove the MCLR from the media.

2.3 Protein phosphatase activity analysis

The activity of PP2A was measured using a serine/threonine phosphatase assay kit from Promega according to the manufacturer's specifications. Briefly, the cells were collected at the indicated time and, before lysis, washed on ice with phosphatase storage buffer (50 mmol/L Tris-HCl, pH 7.5, 0.05% Triton X-100, 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.5 mmol/L phenylmethanesulfonyl fluoride (PMSF), 0.05% (v/v) β-mercaptoethanol, 10% (v/v) glycerol) supplemented with protease inhibitor cocktail for 40 min. To measure phosphatase activity in whole-cell lysates, the free phosphate was first removed from the cell lysates by passage through a Sephadex column. Serine/threonine phosphatase activity in these cell lysates was then measured by their capacity to dephosphorylate a synthetic phosphothreonine peptide, RRA (pT) VA, specific for PP2A. After 30 min of incubation at 37 °C, the free phosphate was determined by measuring the absorbance of the molybdate-malachite green-phosphate complex at 630 nm. The PP2A activity was defined as the number of picomoles of free PO_4 that was generated per min per mg of protein.

2.4 Real-time polymerase chain reaction (PCR)

After the indicated treatments, total RNA was prepared using Trizol (Invitrogen) according to the manufacturer's instructions. A total of 0.5 μg of total RNA was used to synthesize cDNA with an SYBR PrimeScript RT-RCR kit (TaKaRa), according to the manufacturer's protocol. Real-time quantitative PCR was performed using SYBR green PCR core reagents with the ABI 7500 sequence detection system. The following primers were used: Cα (forward, CATC ACCATTCTTCGAGGG; reverse, GGCAGTGAGA GGAAGATAG), Cβ (forward, AGAGGAAATCAC GAAAGCCG; reverse, CAGAATATCTGTCCAT CTAC), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; forward, AGAAGGCTGGGGCTC ATTTG; reverse, AGGGGCCATCCACAGTCTTC). The PCR products of the PP2A subunits were obtained at 95 °C for 30 s, followed by 35 cycles of 95 °C for 15 s, 56 °C for 30 s, and 72 °C for 35 s. The comparative threshold cycle (C_T) method was used to quantify the data using GAPDH as the normalization gene.

2.5 Western blot analysis

Aliquots of the supernatant containing 50 μg of protein were subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the resolved proteins were transferred and incubated with primary antibodies. The primary antibodies were diluted according to the manufacturer's instructions. The membranes were incubated with an HRP-conjugated secondary antibody (1:5000 dilution) and then developed with a chemiluminescence reagent. The quantification of the intensities of the protein bands was performed using Quantity One® 1-D analysis software.

2.6 Immunofluorescence

Cells on coverslips were fixed in 3.7% (v/v) formaldehyde for 10 min at room temperature and blocked for 20 min in blocking buffer (PBS supplemented with 10% FBS) at room temperature. The cells were then incubated with 1 g/L saponin (Sigma) in blocking buffer containing the anti-PP2Ac antibody at room temperature for 1 h. An Alexa^{488}-labeled secondary antibody (Invitrogen) served as a secondary probe and was incubated with the cells for 1 h. The nucleoli were stained with 1 μg/ml DAPI for 10 min at room temperature. For the analysis of co-localization, the images were acquired by a laser-scanning confocal microscope (63×) (Zeiss LSM 510 META) and analyzed using the Zeiss LSM image browser.

2.7 Statistical analysis

Each experiment was performed three times. One-way analysis of variance (ANOVA) was

performed to evaluate the differences between the treatment groups and controls. Statistical significance was defined as $P \leq 0.05$.

3 Results

3.1 Binding of MCLR and PP2Ac

Based on recent studies, a group of hepatocyte uptake transporters, termed organic anion transporting polypeptides (OATPs), were thought to be crucial for the transfer of MCLR into cells (Komatsu *et al*., 2007; Monks *et al*., 2007), and it was questioned whether MCLR could be transported into cultured cell lines without the expression of OATPs. Therefore, the antibody against MCLR was used to confirm the accumulation of MCLR in FL cells. The MCLR in the culture system was carefully removed. As shown in Fig. 1a, the extent of MCLR binding to PP2Ac exhibited dose and time dependencies though a band upper 36 kDa, indicating that a non-specific binding with MCLR antibody was detected. An immunofluorescence assay was later performed by probing for both PP2Ac and MCLR. As shown in Fig. 1b, the images were consistent with previous results and, together, indicated the binding of MCLR and PP2Ac in FL cells. In addition, as shown in Fig. 1c, a band was found approximately 1 kDa above the targeted 36-kDa band for both phosphorylated and total C (in the 10 μmol/L group after 24 h), implicating the existence of MC-conjugated PP2Ac. The increased phosphorylation of PP2Ac was also observed in Fig. 1c, indicating that binding to MCLR impaired PP2Ac's ability to dephosphorylate Tyr^{307}, which resulted in the down-regulation of PP2A activity.

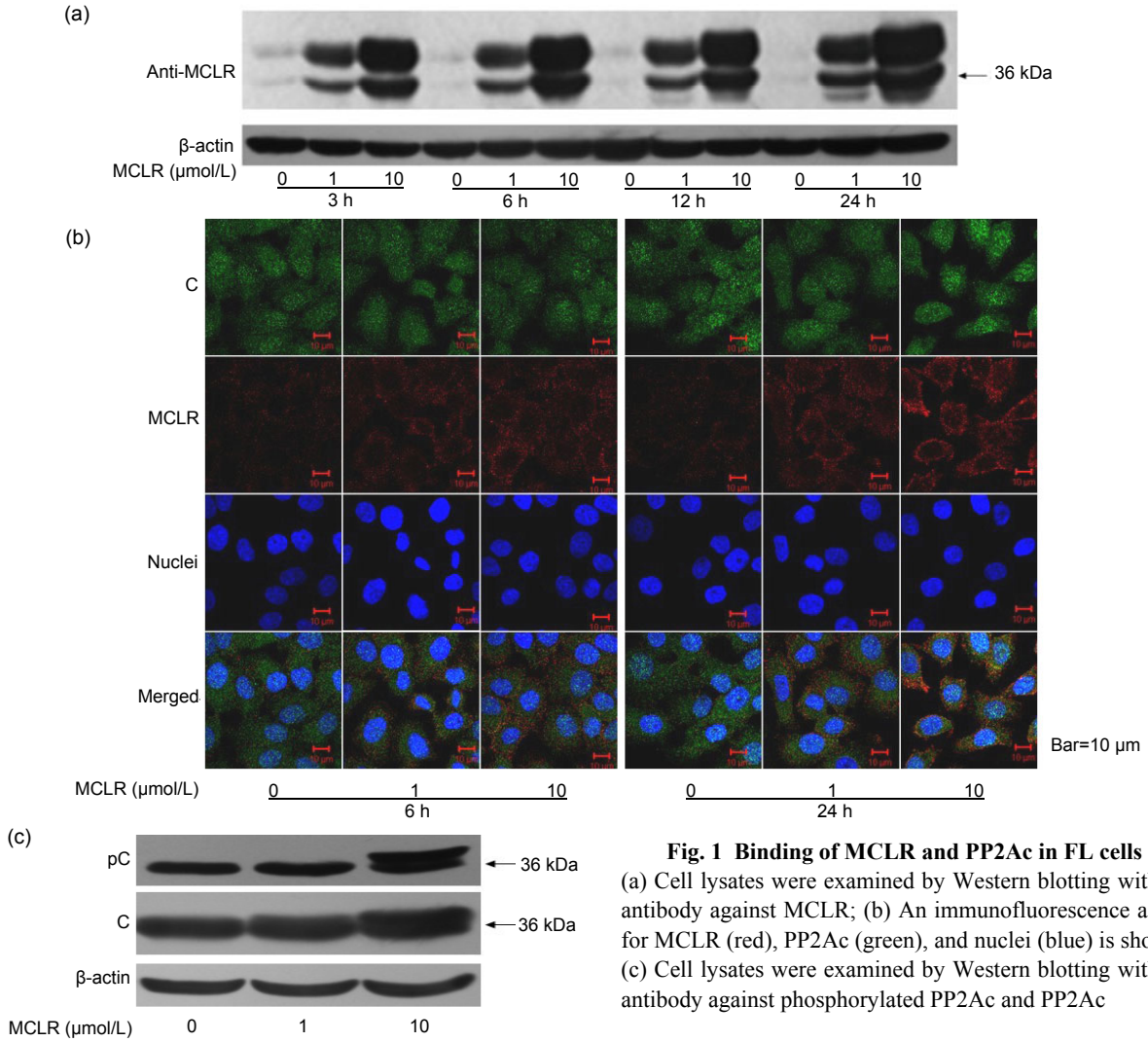

Fig. 1 Binding of MCLR and PP2Ac in FL cells
(a) Cell lysates were examined by Western blotting with an antibody against MCLR; (b) An immunofluorescence assay for MCLR (red), PP2Ac (green), and nuclei (blue) is shown; (c) Cell lysates were examined by Western blotting with an antibody against phosphorylated PP2Ac and PP2Ac

3.2 Changed PP2A activity after exposure to MCLR

To measure the effect of MCLR accumulation on PP2A in cells, FL cells were exposed to different concentrations of MCLR for 6 and 24 h. As shown in Fig. 2, PP2A activity was markedly increased at low concentrations of MCLR for 6 h in the 0.5-μmol/L group, an increase of about 40%. The PP2A activity of the 1.0-μmol/L group was also increased by 31% relative to the control. However, exposure to high concentrations of MCLR for 24 h significantly decreased the activity of PP2A, especially in the 5.0- and 10.0-μmol/L groups.

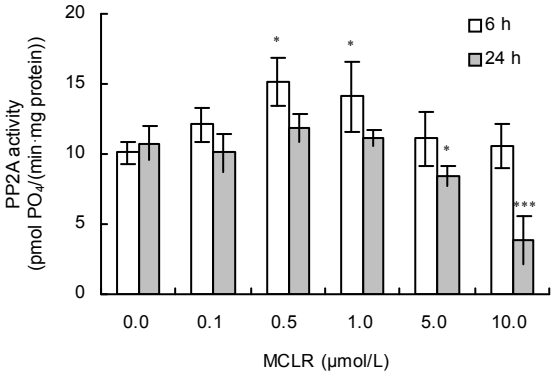

Fig. 2 Changes of PP2A activity after MCLR treatment
Cells were incubated with the indicated concentrations of MCLR for 6 and 24 h, and the activity of PP2A was then measured. The data are indicated as mean±SD obtained from three independent experiments ($^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$)

3.3 Increases of PP2Ac mRNA and protein levels after MCLR treatment

The fact that PP2A is inhibited by MCLR has been well confirmed. Therefore, it is interesting to note the differential responses of FL cells at low-dose exposures of MCLR. Next the PP2Ac mRNA and protein levels, which may be responsible for the increase in PP2A activity, were measured. As shown in Fig. 3a, there was a trend toward increased mRNA levels of C in the MCLR-treated groups compared to the control group, and the protein expression of C also exhibited a corresponding increase with its activity rise in Fig. 3b. In particular, the protein level of C was increased by about 50% in the 1.0-μmol/L group. Based on these results, the increased expression levels of PP2Ac can be attributed to the up-regulation of PP2A activity by a low dose of MCLR.

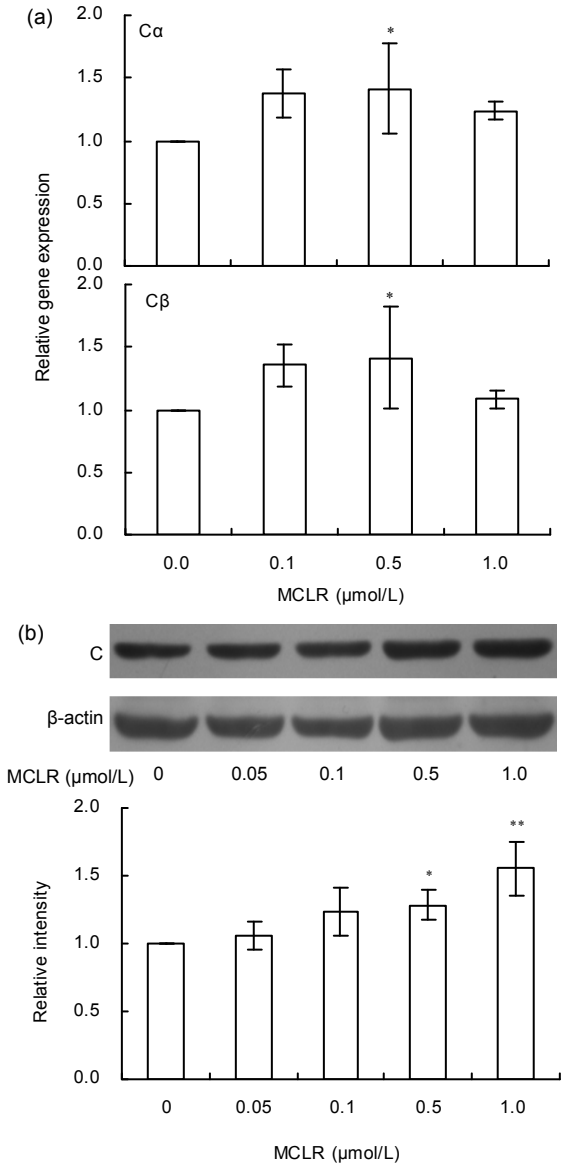

Fig. 3 Increased expression levels of PP2Ac mRNA and protein after MCLR treatment
FL cells were treated with MCLR for 6 h. (a) The mRNA levels of C were examined by real-time PCR; (b) Western blotting revealed increased protein levels of C

3.4 Alternation of acetylated tubulin dependent on PP2A activity

Merrick *et al.* (1997) reported that PP2A activity is implicated in MT instability, particularly the post-translational modifications of tubulin (α-tubulin acetylation and detyrosination). To understand MT responses to the alteration of PP2A activity in FL cells, the post-translational modifications of tubulin were also investigated after exposure to MCLR. As shown in Fig. 4, the level of acetylated tubulin was

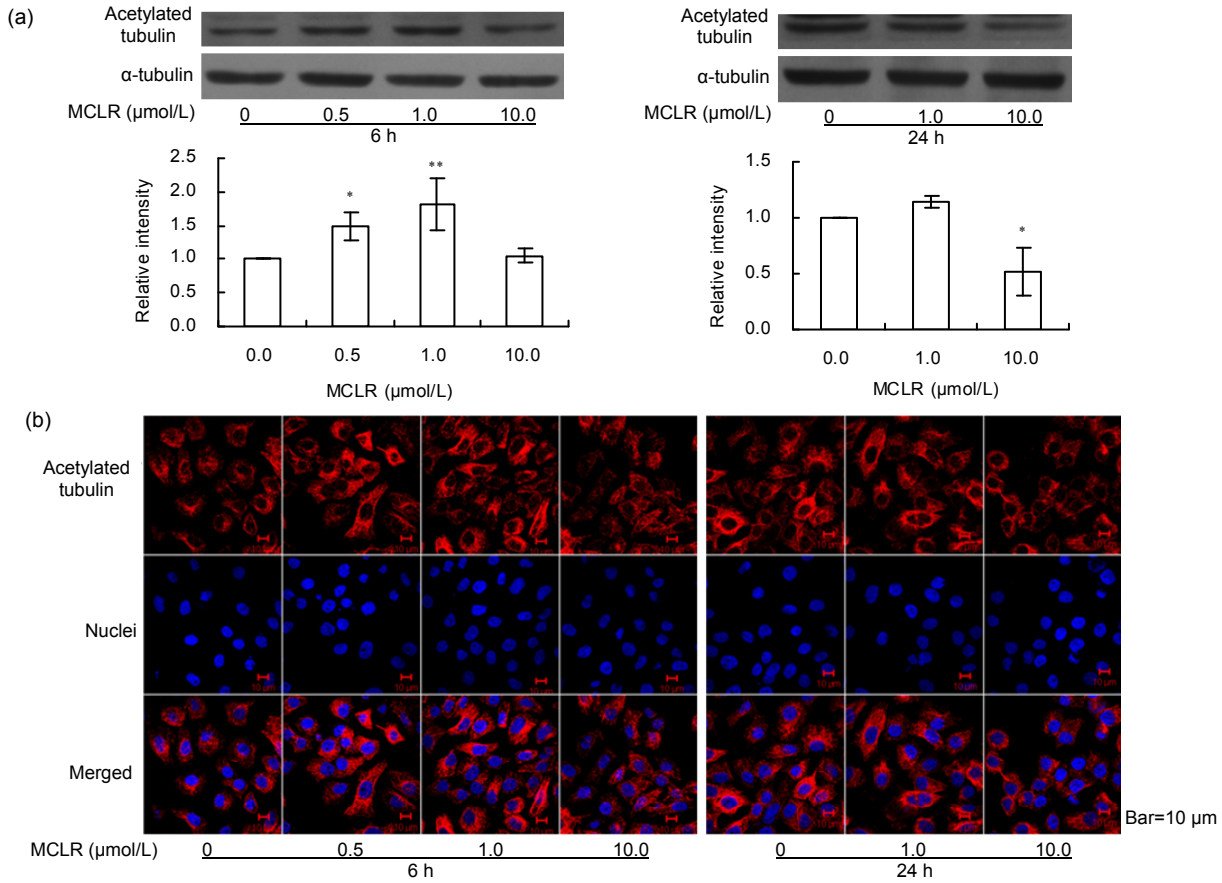

Fig. 4 Effect of changed PP2A activity on the level of acetylated tubulin

FL cells were treated with MCLR for 6 and 24 h. The level of acetylated tubulin was detected by Western blotting (a) and immunofluorescence (b). An immunofluorescence assay of acetylated tubulin (red) and nuclei (blue) is shown in (b)

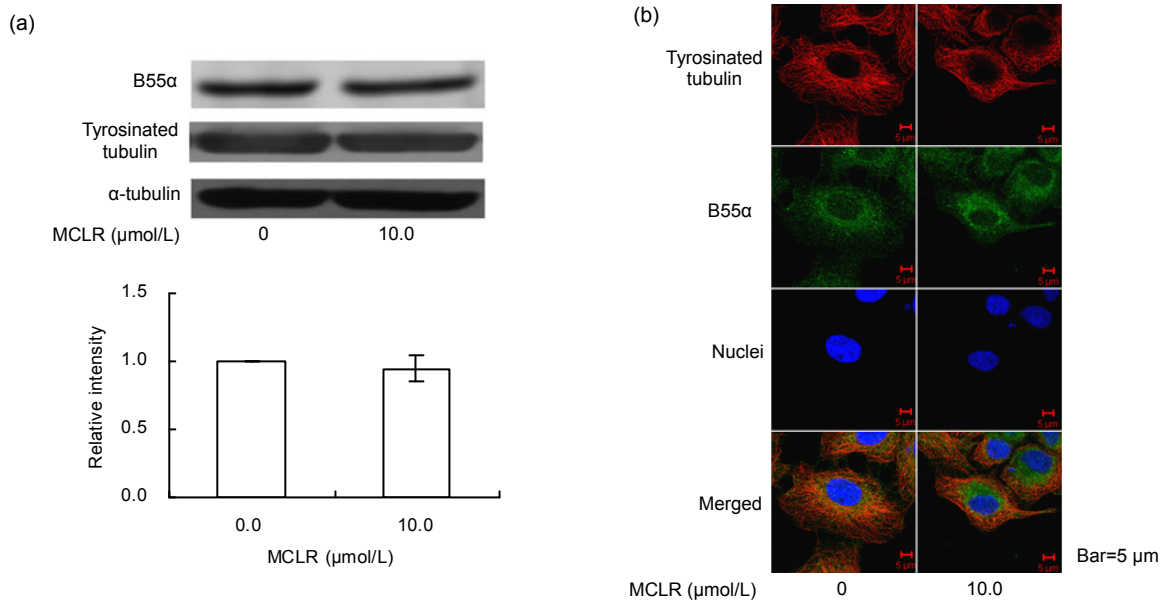

Fig. 5 Impairment of the ability of tyrosinated tubulin to associate with the B55α subunit in FL cells after exposure to MCLR

(a) Western blotting was performed to detect the levels of B55α and tyrosinated tubulin; (b) The co-localization of B55α (green) and tyrosinated tubulin (red) is shown by an immunofluorescence assay

up-regulated after a low-dose and short-time exposure, due to the increased activity of PP2A. A loss of acetylated tubulin was observed in cells treated with a high dose of MCLR, in which PP2A activity was significantly inhibited. Based on these results, the acetylation of tubulin is dependent on the activity of PP2A.

3.5 Reduced binding of tyrosinated tubulin and B55α after MCLR treatment

To further define the involvement of PP2A in the MCLR-induced depolymerization of MTs, regulatory subunit B55α was chosen for further evaluation. As shown in Fig. 5a, Western blotting analysis revealed that the total expression level of B55α did not change relative to the control, and the level of tyrosinated tubulin also did not change after MCLR treatment for 24 h. However, as clearly shown in Fig. 5b, there was shrinkage of the tyrosinated tubulin, and then some of the B55α subunit (green) no longer bound to the tyrosinated tubulin (red) in FL cells, indicating that exposure to MCLR decreased the binding of B55α and tyrosinated tubulin.

4 Discussion

Given the various functions of PP2A in vivo, much more can still be learned about the exact response of PP2A to MCLR exposure. Thus, in the present study, immortalized (but not tumorigenic) human amniotic epithelial cells were exposed to MCLR to observe any alterations in PP2A.

In the present study, the opposing alterations in PP2A activity induced by lower and higher doses of MCLR at different exposure time are termed hormesis, which is a dose-response phenomenon that is characterized by low-dose stimulation and high-dose inhibition (Calabrese and Baldwin, 2002). Previous in vivo and in vitro studies support the contrasting responses to MCLR exposure: low doses of MCLR appear to increase cell survival and proliferation, but higher doses of the toxin reduce cell viability (Sekijima *et al*., 1999; Gehringer, 2004). Although the exact mechanism has not been revealed, the striking alternation of PP2A activity may be one of the key features.

Furthermore, MCLR has widely been thought to be an inhibitor of PP2A. However, a notable result of the present study is the up-regulation of PP2A phosphatase activity following exposure to MCLR. Furthermore, as shown in Fig. 3, a visible increase in PP2Ac mRNA and protein levels coincided with the increased PP2A activity. Although the protein levels of PP2Ac in cells are under strict auto-regulatory control (Baharians and Schonthal, 1998), these findings indicate the presence of response machinery, where exposure to MCLR generates a signal that leads to an increase in the transcription and translation of the C subunit. The level of the PP2Ac protein in FL cells was increased by about 50%, coinciding with the observation that the compensation stimulation in hormesis is modest and the range is typically in 30%–60% (Calabrese, 2008). The present results seem to indicate that PP2Ac levels can be quite dynamic.

It has been well established that the post-translational modifications of α-tubulin (including acetylation and tyrosination) play key regulatory roles in MT stabilization (Piperno *et al*., 1987; Matsuyama *et al*., 2002). Acetylation occurs after MT assembly, and depolymerized tubulin is rapidly deacetylated (Sasse and Gull, 1988; Matsuyama *et al*., 2002). As shown in Fig. 4, the levels of acetylated MTs changed with the inhibition of PP2A activity, indicating that PP2A is probably required for regulating the acetylation of tubulin. In particular, it has been proven that histone deacetylase 6 (HDAC6), which is mainly responsible for deacetylating tubulin (Hubbert *et al*., 2002), is bound directly to PP1 (Brush *et al*., 2004). Although there is no evidence for an association between PP2A and HDAC6, our data suggest that PP2A facilitates the recruitment of HDAC6 to modulate MT dynamics. However, the exact mechanism needs further investigation.

Earlier studies performed with okadaic acid (OA) have indicated that the inhibition of PP2A activity induces the selective destruction of detyrosinated MTs (Gurland and Gundersen, 1993; Hamm-Alvarez *et al*., 1996; Merrick *et al*., 1997), and B55α silencing promotes MT destabilization (Nunbhakdi-Craig *et al*., 2007). These data demonstrate the important role of B55α-containing PP2A in regulating MT stability. However, the exact molecular mechanism of MCLR-induced MT destabilization has not been fully elucidated. The immunofluorescence results in Fig. 5 revealed that the severely impaired B55α binding to tyrosinated tubulin may be one reason, which may

suggest that the decreased regulation of tyrosinated tubulin by PP2A induces the increased depolymerization of MT. Together, these results indicate that B55α-containing PP2A is an important contributor to MT destabilization induced by MCLR.

In summary, the results of the current study reveal, for the first time, that exposure to MCLR increases PP2A phosphatase activity by up-regulating the expression of C at both the mRNA and protein levels. In addition, exposure to MCLR impairs the ability of B55α-containing PP2A to bind to tyrosinated tubulin, suggesting that PP2A is involved in the MT destabilization induced by MCLR. In particular, the ability of MCLR to increase the phosphatase activity of PP2A in FL cells may generate new avenues to further investigation of the mechanism of MCLR toxicity.

References

Baharians, Z., Schonthal, A.H., 1998. Autoregulation of protein phosphatase type 2A expression. *J. Biol. Chem.*, **273**(30):19019-19024. [doi:10.1074/jbc.273.30.19019]

Brush, M.H., Guardiola, A., Connor, J.H., Yao, T.P., Shenolikar, S., 2004. Deacetylase inhibitors disrupt cellular complexes containing protein phosphatases and deacetylases. *J. Biol. Chem.*, **279**(9):7685-7691. [doi:10.1074/jbc.M310997200]

Calabrese, E.J., 2008. Hormesis: why it is important to toxicology and toxicologists. *Environ. Toxicol. Chem.*, **27**(7):1451-1474. [doi:10.1897/07-541.1]

Calabrese, E.J., Baldwin, L.A., 2002. Applications of hormesis in toxicology, risk assessment and chemotherapeutics. *Trends Pharmacol. Sci.*, **23**(7):331-337. [doi:10.1016/S0165-6147(02)02034-5]

Chen, J., Martin, B.L., Brautigan, D.L., 1992. Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation. *Science*, **257**(5074):1261-1264. [doi:10.1126/science.1325671]

Chen, T., Wang, Q., Cui, J., Yang, W., Shi, Q., Hua, Z., Ji, J., Shen, P., 2005. Induction of apoptosis in mouse liver by microcystin-LR: a combined transcriptomic, proteomic, and simulation strategy. *Mol. Cell. Proteomics*, **4**(7):958-974. [doi:10.1074/mcp.M400185-MCP200]

Chen, W., Possemato, R., Campbell, K.T., Plattner, C.A., Pallas, D.C., Hahn, W.C., 2004. Identification of specific PP2A complexes involved in human cell transformation. *Cancer Cell*, **5**(2):127-136. [doi:10.1016/S1535-6108(04)00026-1]

Clark, S.P., Davis, M.A., Ryan, T.P., Searfoss, G.H., Hooser, S.B., 2007. Hepatic gene expression changes in mice associated with prolonged sublethal microcystin exposure. *Toxicol. Pathol.*, **35**(4):594-605. [doi:10.1080/01926230701383210]

Ding, W.X., Shen, H.M., Zhu, H.G., Ong, C.N., 1998. Studies on oxidative damage induced by cyanobacteria extract in primary cultured rat hepatocytes. *Environ. Res.*, **78**(1):12-18. [doi:10.1006/enrs.1998.3843]

Ding, W.X., Shen, H.M., Ong, C.N., 2000a. Critical role of reactive oxygen species and mitochondrial permeability transition in microcystin-induced rapid apoptosis in rat hepatocytes. *Hepatology*, **32**(3):547-555. [doi:10.1053/jhep.2000.16183]

Ding, W.X., Shen, H.M., Ong, C.N., 2000b. Microcystic cyanobacteria extract induces cytoskeletal disruption and intracellular glutathione alteration in hepatocytes. *Environ. Health Perspect.*, **108**(7):605-609. [doi:10.1289/ehp.00108605]

dos S. Vieira, J.M., de P. Azevedo, M.T., de Oliveira Azevedo, S.M., Honda, R.Y., Corrêa, B., 2005. Toxic cyanobacteria and microcystin concentrations in a public water supply reservoir in the Brazilian Amazonia region. *Toxicon*, **45**(7):901-909. [doi:10.1016/j.toxicon.2005.02.008]

Fogh, J., Lund, R.O., 1957. Continuous cultivation of epithelial cell strain (FL) from human amniotic membrane. *Proc. Soc. Exp. Biol. Med.*, **94**(3):532-537.

Fu, W.Y., Chen, J.P., Wang, X.M., Xu, L.H., 2005. Altered expression of p53, Bcl-2 and Bax induced by microcystin-LR in vivo and in vitro. *Toxicon*, **46**(2):171-177. [doi:10.1016/j.toxicon.2005.03.021]

Fu, W.Y., Yu, Y.N., Xu, L.H., 2009. Identification of temporal differentially expressed protein responses to microcystin in human amniotic epithelial cells. *Chem. Res. Toxicol.*, **22**(1):41-51. [doi:10.1021/tx800396p]

Gehringer, M.M., 2004. Microcystin-LR and okadaic acid-induced cellular effects: a dualistic response. *FEBS Lett.*, **557**(1-3):1-8. [doi:10.1016/S0014-5793(03)01447-9]

Grochola, L.F., Vazquez, A., Bond, E.E., Wurl, P., Taubert, H., Muller, T.H., Levine, A.J., Bond, G.L., 2009. Recent natural selection identifies a genetic variant in a regulatory subunit of protein phosphatase 2A that associates with altered cancer risk and survival. *Clin. Cancer Res.*, **15**(19):6301-6308. [doi:10.1158/1078-0432.CCR-09-0797]

Gurland, G., Gundersen, G.G., 1993. Protein phosphatase inhibitors induce the selective breakdown of stable microtubules in fibroblasts and epithelial cells. *PNAS*, **90**(19):8827-8831. [doi:10.1073/pnas.90.19.8827]

Hamm-Alvarez, S.F., Wei, X., Berndt, N., Runnegar, M., 1996. Protein phosphatases independently regulate vesicle movement and microtubule subpopulations in hepatocytes. *Am. J. Physiol.*, **271**(3 Pt 1):C929-C943.

Hoeger, S.J., Hitzfeld, B.C., Dietrich, D.R., 2005. Occurrence and elimination of cyanobacterial toxins in drinking water treatment plants. *Toxicol. Appl. Pharmacol.*, **203**(3):231-242. [doi:10.1016/j.taap.2004.04.015]

Hu, Z., Chen, H., Li, Y., Gao, L., Sun, C., 2002. The expression of bcl-2 and bax genes during microcystin induced liver tumorigenesis. *Chin. J. Prev. Med.*, **36**(4):239-242 (in Chinese).

Huang, P., Zheng, Y.F., Xu, L.H., 2008. Oral administration of cyanobacterial bloom extract induced the altered expression of the PP2A, Bax, and Bcl-2 in mice. *Environ.*

Toxicol., **23**(6):688-693. [doi:10.1002/tox.20374]

Huang, P., Zheng, Q., Xu, L.H., 2011. The apoptotic effect of oral administration of microcystin-RR on mice liver. *Environ. Toxicol.*, **26**(5):443-452. [doi:10.1002/tox.20570]

Hubbert, C., Guardiola, A., Shao, R., Kawaguchi, Y., Ito, A., Nixon, A., Yoshida, M., Wang, X.F., Yao, T.P., 2002. HDAC6 is a microtubule-associated deacetylase. *Nature*, **417**(6887):455-458. [doi:10.1038/417455a]

Humpage, A.R., Hardy, S.J., Moore, E.J., Froscio, S.M., Falconer, I.R., 2000. Microcystins (cyanobacterial toxins) in drinking water enhance the growth of aberrant crypt foci in the mouse colon. *J. Toxicol. Environ. Health A*, **61**(3): 155-165. [doi:10.1080/00984100050131305]

Ito, E., Kondo, F., Terao, K., Harada, K., 1997. Neoplastic nodular formation in mouse liver induced by repeated intraperitoneal injections of microcystin-LR. *Toxicon*, **35**(9):1453-1457. [doi:10.1016/S0041-0101(97)00026-3]

Janssens, V., Goris, J., 2001. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem. J.*, **353**(3): 417-439. [doi:10.1042/0264-6021:3530417]

Jochimsen, E.M., Carmichael, W.W., An, J.S., Cardo, D.M., Cookson, S.T., Holmes, C.E., Antunes, M.B., de Melo Filho, D.A., Lyra, T.M., Barreto, V.S., *et al*., 1998. Liver failure and death after exposure to microcystins at a hemodialysis center in Brazil. *N. Engl. J. Med.*, **338**(13): 873-878. [doi:10.1056/NEJM199803263381304]

Komatsu, M., Furukawa, T., Ikeda, R., Takumi, S., Nong, Q., Aoyama, K., Akiyama, S., Keppler, D., Takeuchi, T., 2007. Involvement of mitogen-activated protein kinase signaling pathways in microcystin-LR-induced apoptosis after its selective uptake mediated by OATP1B1 and OATP1B3. *Toxicol. Sci.*, **97**(2):407-416. [doi:10.1093/toxsci/kfm054]

Li, H., Xie, P., Li, G., Hao, L., Xiong, Q., 2009. 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 male Wistar rats injected i.v. with toxins. *Toxicon*, **53**(1):169-175. [doi:10.1016/j.toxicon.2008.10.027]

Liu, G., Shang, Y., Yu, Y., 2006. Induced endoplasmic reticulum (ER) stress and binding of over-expressed ER specific chaperone GRP78/BiP with dimerized epidermal growth factor receptor in mammalian cells exposed to low concentration of *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine. *Mutat. Res.*, **596**(1-2):12-21. [doi:10.1016/j.mrfmmm.2005.10.015]

Malbrouck, C., Trausch, G., Devos, P., Kestemont, P., 2003. Hepatic accumulation and effects of microcystin-LR on juvenile goldfish *Carassius auratus* L. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.*, **135**(1):39-48. [doi:10.1016/S1532-0456(03)00047-4]

Matsuyama, A., Shimazu, T., Sumida, Y., Saito, A., Yoshimatsu, Y., Seigneurin-Berny, D., Osada, H., Komatsu, Y., Nishino, N., Khochbin, S., *et al*., 2002. In vivo destabilization of dynamic microtubules by HDAC6-mediated deacetylation. *EMBO J.*, **21**(24):6820-6831. [doi:10.1093/emboj/cdf682]

McDermott, C.M., Nho, C.W., Howard, W., Holton, B., 1998. The cyanobacterial toxin, microcystin-LR, can induce apoptosis in a variety of cell types. *Toxicon*, **36**(12): 1981-1996. [doi:10.1016/S0041-0101(98)00128-7]

Merrick, S.E., Trojanowski, J.Q., Lee, V.M., 1997. Selective destruction of stable microtubules and axons by inhibitors of protein serine/threonine phosphatases in cultured human neurons. *J. Neurosci.*, **17**(15):5726-5737.

Monks, N.R., Liu, S., Xu, Y., Yu, H., Bendelow, A.S., Moscow, J.A., 2007. Potent cytotoxicity of the phosphatase inhibitor microcystin LR and microcystin analogues in OATP1B1- and OATP1B3-expressing HeLa cells. *Mol. Cancer Ther.*, **6**(2):587-598. [doi:10.1158/1535-7163.MCT-06-0500]

Moreno, I., Pichardo, S., Jos, A., Gomez-Amores, L., Mate, A., Vazquez, C.M., Camean, A.M., 2005. Antioxidant enzyme activity and lipid peroxidation in liver and kidney of rats exposed to microcystin-LR administered intraperitoneally. *Toxicon*, **45**(4):395-402. [doi:10.1016/j.toxicon.2004.11.001]

Nunbhakdi-Craig, V., Schuechner, S., Sontag, J.M., Montgomery, L., Pallas, D.C., Juno, C., Mudrak, I., Ogris, E., Sontag, E., 2007. Expression of protein phosphatase 2A mutants and silencing of the regulatory B α subunit induce a selective loss of acetylated and detyrosinated microtubules. *J. Neurochem.*, **101**(4):959-971. [doi:10.1111/j.1471-4159.2007.04503.x]

Pinho, G.L., Moura da Rosa, C., Yunes, J.S., Luquet, C.M., Bianchini, A., Monserrat, J.M., 2003. Toxic effects of microcystins in the hepatopancreas of the estuarine crab *Chasmagnathus granulatus* (Decapoda, Grapsidae). *Comp. Biochem. Physiol. C Toxicol. Pharmacol.*, **135**(4): 459-468. [doi:10.1016/S1532-0456(03)00147-9]

Piperno, G., LeDizet, M., Chang, X.J., 1987. Microtubules containing acetylated alpha-tubulin in mammalian cells in culture. *J. Cell Biol.*, **104**(2):289-302. [doi:10.1083/jcb.104.2.289]

Robinson, N.A., Miura, G.A., Matson, C.F., Dinterman, R.E., Pace, J.G., 1989. Characterization of chemically tritiated microcystin-LR and its distribution in mice. *Toxicon*, **27**(9):1035-1042. [doi:10.1016/0041-0101(89)90154-2]

Sasse, R., Gull, K., 1988. Tubulin post-translational modifications and the construction of microtubular organelles in *Trypanosoma brucei*. *J. Cell Sci.*, **90**(Pt 4):577-589.

Sekijima, M., Tsutsumi, T., Yoshida, T., Harada, T., Tashiro, F., Chen, G., Yu, S.Z., Ueno, Y., 1999. Enhancement of glutathione *S*-transferase placental-form positive liver cell foci development by microcystin-LR in aflatoxin B1-initiated rats. *Carcinogenesis*, **20**(1):161-165. [doi:10.1093/carcin/20.1.161]

Shen, J., Wu, M., Yu, Y., 2006. Proteomic profiling for cellular responses to different concentrations of *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine. *J. Proteome Res.*, **5**(2):385-395. [doi:10.1021/pr0502875]

Tolstykh, T., Lee, J., Vafai, S., Stock, J.B., 2000. Carboxyl methylation regulates phosphoprotein phosphatase 2A by

controlling the association of regulatory B subunits. *EMBO J.*, **19**(21):5682-5691. [doi:10.1093/emboj/19.21.5682]

Vazquez, A., Kulkarni, D., Grochola, L.F., Bond, G.L., Barnard, N., Toppmeyer, D., Levine, A.J., Hirshfield, K.M., 2011. A genetic variant in a PP2A regulatory subunit encoded by the *PPP2R2B* gene associates with altered breast cancer risk and recurrence. *Int. J. Cancer*, **128**(10):2335-2343. [doi:10.1002/ijc.25582]

Wu, M., Shen, J., Zhan, J., Yu, Y., 2006. dUTP Pyrophosphatase, its appearance in extracellular compartment may serve as a potential biomarker for *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine exposure in mammalian cells. *Proteomics*, **6**(10):3001-3007. [doi:10.1002/pmic.200500767]

Xing, M.L., Wang, X.F., Xu, L.H., 2008. Alteration of proteins expression in apoptotic FL cells induced by MCLR. *Environ. Toxicol.*, **23**(4):451-458. [doi:10.1002/tox.20355]

Xing, Y., Xu, Y., Chen, Y., Jeffrey, P.D., Chao, Y., Lin, Z., Li, Z., Strack, S., Stock, J.B., Shi, Y., 2006. Structure of protein phosphatase 2A core enzyme bound to tumor-inducing toxins. *Cell*, **127**(2):341-353. [doi:10.1016/j.cell.2006.09.025]

Xu, Y., Xing, Y., Chen, Y., Chao, Y., Lin, Z., Fan, E., Yu, J.W., Strack, S., Jeffrey, P.D., Shi, Y., 2006. Structure of the protein phosphatase 2A holoenzyme. *Cell*, **127**(6):1239-1251. [doi:10.1016/j.cell.2006.11.033]

Yoshizawa, S., Matsushima, R., Watanabe, M.F., Harada, K., Ichihara, A., Carmichael, W.W., Fujiki, H., 1990. Inhibition of protein phosphatases by microcystins and nodularin associated with hepatotoxicity. *J. Cancer Res. Clin. Oncol.*, **116**(6):609-614. [doi:10.1007/BF01637082]

Yu, S.Z., 1995. Primary prevention of hepatocellular carcinoma. *J. Gastroenterol. Hepatol.*, **10**(6):674-682. [doi:10.1111/j.1440-1746.1995.tb01370.x]

Zhu, X., Xing, M., Lou, J., Wang, X., Fu, W., Xu, L., 2007. Apoptotic related biochemical changes in human amnion cells induced by tributyltin. *Toxicology*, **230**(1):45-52. [doi:10.1016/j.tox.2006.10.026]