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Combined seven miRNAs for early hepatocellular carcinoma detection with chronic low-dose exposure to microcystin-LR in mice

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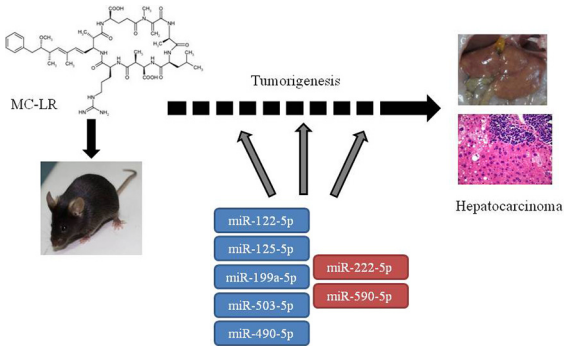
HIGHLIGHTS

- Histopathological changes of cancer were observed in mice of MC-LR-induced HCC.
- miR-122-5p, 125-5p, 199a-5p, and 503-5p were reduced at similar times and doses.
- Combined marker set of seven miRNAs detects early hepatocellular carcinoma.

GRAPHICAL ABSTRACT



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ABSTRACT

Aberrant miRNA expression has been detected in various tumor tissues, which may be considered as a marker for early cancer diagnosis. One miRNA has multiple downstream target genes, which can be regulated by multiple upstream other miRNAs. Hence, this dynamic regulation is likely characterized by volatility, and thus, finding the appropriate time point for tests becomes essential for the use of miRNAs as an early marker of tumor diagnosis. In this study, we established a chronic liver cancer progression model in mice by using low doses of the harmful substance microcystin-LR (MC-LR). On the basis of miRNAs microarray assay, we further tested seven miRNAs that showed characteristic expression changes in pre-hepatocarcinogenesis. Our results showed that the levels of four miRNAs (miR-122-5p, miR-125-5p, miR-199a-5p, and miR-503-5p) decreased dramatically, whereas those of two miRNAs (miR-222-5p and miR-590-5p) increased significantly in the early stages, which were all accompanied by an increase in atypia of hepatocytes. MiR-490-5p was a sensitive molecular, suitable only for evaluation of pathological changes in young mice. Therefore the combination the seven of miRNAs for a set may prove to be an effective method in healthy assessment of environmental toxicants for detection of hepatocarcinogenesis caused by hazardous materials.

1. Introduction

Microcystins (MCs) are a group of closely related cyclic heptapeptides comprising >80 variants that are produced by freshwater cyanobacteria (Eriksson et al., 1989; Ohta et al., 1992; Li et al., 2014).

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Chronic exposure to MCs remains the most common means of intoxication for animals and humans. In addition, epidemiologic studies suggested that the MCs exposure with drinking water was associated with a high risk for cancer and may be responsible for the increased incidence of HCC in some areas of China where people drink water from ponds, lakes, and shallow wells (Ueno et al., 1996; Zheng et al., 2017). In our previous studies, we confirmed that the chronic exposure to microcystin-LR (MC-LR), which is the most common endotoxic variant, can cause liver cell malignant transformation *in vitro* (Xu et al., 2012).

MicroRNAs (miRNAs) can regulate diverse cellular functions at the post-transcriptional level and have important roles in a wide variety of physiological and pathological cellular processes (Bartel, 2004; Carthew, 2006; Krutovskikh and Herceg, 2010; Mansoori et al., 2017). Aberrant miRNAs expression has been associated with various human diseases, especially tumors (Dufresne et al., 2017). A considerable body of evidence supports the hypothesis that miRNAs regulate cancer development and are involved in environmental pollutant- and toxicant-induced carcinogenesis. All investigated human cancers, including hepatocarcinoma (HCC), are characterized by globally abnormal miRNA expression patterns (Pollutri et al., 2016; Reichl and Mikulits, 2016; Xu et al., 2016). Some studies have pointed out that certain specific miRNAs may even be detected in the serum of cancer patients, including those with HCC (El-Abd et al., 2015; Dufresne et al., 2017). Previous research indicated that miRNAs expression changes stimulate the development of cancer. Hence, the analysis of miRNAs expression at the early stages of cancer can be useful as a tool for early monitoring of tumorigenesis, especially for healthy assessment of environmental toxicants (Inokawa et al., 2016; Lin et al., 2017).

Hepatocarcinogenesis involves a complex, multistep process and is tightly linked to chronic liver damage. However, the literature shows that the activation of oncogenic pathways and the dysfunction of tumor suppressor genes in human HCC appear more heterogeneous than in other cancers. Therefore, its mechanism still remains to be addressed. Recent studies suggested that the alterations of miRNAs expression play an important role in the pathogenesis of HCC (Gyugos et al., 2014; Shen et al., 2016). Our preliminary examinations also indicate the presence of abnormal miRNAs expression in MC-LR-induced phenotypic transformation *in vitro*. These data indicated that the characteristic miRNAs alterations could be used as molecular targets for environmental toxicants for health. However, it is more important and also complex that these valuable novel properties and potential be used and confirmed *in vivo* studies. On the other hand, the role of miRNAs in tumorigenesis is paradoxical. One miRNA can control multiple genes, but it can also be regulated by more than one gene. That is to say, establishing the right time point is of crucial importance for the application of miRNAs as an early marker of tumor diagnosis. Some miRNAs with a close relationship with HCC were notably altered by different durations and by diverse doses of MC-LR exposure. Then, the expression of characteristic miRNAs, such as miR-122-5p, miR-125-5p, miR-199a-5p, and miR-503-5p, could be used as biomarkers for the healthy assessment of environmental toxicants in water, and for the development of miRNA-based strategies for prevention and treatment of HCC.

2. Material and methods

2.1. Chemicals

MC-LR [purity of ≥95%, by high-performance liquid chromatography (HPLC)] was purchased from Enzo Life Science (Farmingdale, NY, USA). All other chemicals were of analytical grade and provided by common commercial suppliers.

2.2. Animals

The experiments were performed on male C57BL/6 mice (6–8-week old), provided by the Model Animal Research Center of Nanjing University (Nanjing, Jiangsu, China). All mice were acclimated to the environmental conditions for seven days before the toxicological experiment was performed. The animals were housed in a temperature- and humidity-controlled room (21 ± 2 °C, 55 ± 5% humidity, specific-pathogen-free) with a 12-h light/dark cycle. Water and rodent chow were available *ad libitum*. A total of 280 mice were randomly divided into the following seven groups ($n = 40$ per group): six treatment groups (exposure to concentrations of MC-LR in the drinking water of 1, 5, 10, 20, 40, and 80 μg/L, respectively) and one control group. The animals in each of the groups were subjected to MC-LR exposure for 3, 6, 9, and 12 months, respectively; 10 animals were used in each of the examined exposure times. The animals were sacrificed by CO_2 asphyxiation at the end of the MC-LR exposure treatment, including their matched control. Then, the liver tissues were immediately excised and weighed. A part of the liver tissue (4 mm × 4 mm × 4 mm) from each animal was fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) (4% PFA), whereas the residual tissue was kept at −80 °C. All procedures of animal right were approved by the Ethics Committee for Animal Research of Nanjing University.

2.3. Histological observations

Animals were euthanized by CO_2 asphyxiation before decapitation for sample collection. Liver tissues were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) (4% PFA) for 24 h, then trimmed and embedded in paraffin. Four-μm-thick sections were stained with hematoxylin and eosin (H&E). Further, ten 10× light microscopic fields were examined on each section for each liver. The presence of HCC was evaluated by two pathologists.

2.4. Aminotransferase activity determination

Serum alanine aminotransferase (sALT) and serum aspartate aminotransferase (sAST) levels, which are markers for hepatotoxicity, were determined using an automatic analyzer (Hitachi 7600-020, Japan).

2.5. Screening for differentially expressed miRNAs with microarray technology

Total RNAs, including microRNA, was extracted using the mirVana™ microRNA isolation kit (Applied Biosystem, Foster City, CA, USA) according to the manufacturer's instructions to ensure the recovery of small RNA (smaller than 200 nt) as well as larger RNAs. The liver total RNA was labeled using a mercury Hy3™/Hy5™ labeling kit and hybridized on a miRCURY™ LNA Array kit (v. 11.0; Exiqon, Vedbaek, Denmark). The arrays had quadruple-spotted LNA probes against 96% of the 885 human miRNAs in the miRBase 13.0 registry. Signal intensities were normalized using the global Lowess regression algorithm. For subsequent analysis, the log2 of the background-subtracted and normalized median spot intensities of the ratios (fold-change values) from the channel (Hy3) was used.

2.6. Confirmation of the different expression miRNAs with quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The total RNAs were extracted from the liver tissues of the experimental animals. Using the TaqMan MicroRNA Assay Kit, 3 μg of total RNA from each sample was taken to perform the reverse transcription reaction according to the manufacturer's instructions. The resulting cDNA was semi-quantitatively amplified in 45 cycles on an ABI StepOne Plus Real-Time PCR System, using TaqMan Universal PCR Master Mix No Amp Erase UNG and TaqMan MicroRNA Assays for miR-26a-5p, miR-