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Microcystin-LR promotes migration via the cooperation between microRNA-221/PTEN and STAT3 signal pathway in colon cancer cell line DLD-1

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ABSTRACT

Previous researches have reported that microcystin-LR (MC-LR) contributes to the progression of multiple types of carcinomas including colon cancer; however, the underlying molecular mechanisms remain unclear and require in-depth investigation. Here, the colon cell line DLD-1 was arranged for the analysis by the microRNA microarray which was associated with the cancer metastasis after MC-LR exposure. 31 human microRNAs were differentially expressed, including miR-221, which targeted 3′-UTR of PTEN mRNA and PTEN level was down-regulated by MC-LR treatment. Besides, MC-LR also induced the phosphorylation of STAT3, which can be reversed by adding miR-221 inhibitor and PTEN expression plasmid. Furthermore, miR-221 inhibitor, STAT3 siRNA and PTEN expression plasmid could reverse the effects of MC-LR induced migration with the accumulation of β-catenin in nuclei. In conclusion, our study suggested that MC-LR promoted the progression of colon carcinoma, at least in part, by regulating the expression miR-221, PTEN and STAT3 phosphorylation, which offers a novel perspective to understand the connection between MC-LR and colon cancer.

1. Introduction

Cyanobacterial bloom is one of the most noteworthy water pollution in environmental governance. As the products of cyanobacteria, Microcystins (MCs) are a group of cyclic peptide toxins and over 80 congeners have been identified to date (Xing et al., 2008). Microcystin-LR (MC-LR) is the most abundant and toxic variant accounting for 46–99.8% of the total MCs in the natural waters (Zhou et al., 2014). MC-LR leads to hyperphosphorylation of numberous proteins in cells via the specific inhibition of serine/threonine protein phosphatases 1 and 2A (PP1, PP2A), which regulate rearrangements of filamentous actin and microtubule in lung cancer cells (Wang et al., 2017). Epidemiological studies have shown that MC-LR may lead to a high incidence of colorectal cancer (Zhou et al., 2002) and liver cancer in China (Ueno et al., 1996). In addition, our previous work has indicated that MC-LR contributes to invasion and migration of tumor cells through various target proteins, such as MMPs family protein MMP-2, MMP-9 and MMP-13 (Zhang et al., 2012; Miao et al., 2015). So far, little is known about the effect of MC-LR on miRNA in cancer metastasis.

MicroRNA (miRNA) is a small type of non-coding RNA with 18–22 nucleotides, which regulates at post-transcriptional level and silences a broad set of target genes (Ma et al., 2014). The abnormal expression of miRNA plays an essential role in cancer formation and progression. For instance, miR-221, an oncogenic miRNA, which belongs to the miR-221/222 family, is involved in cancer invasion and migration multiple cancers including colorectal cancer, renal cell carcinoma, luminal breast cancer (Dentelli et al., 2014; Qin and Luo, 2014; Lu et al., 2015). The high expression of miR-221 could directly inhibit the target genes such as PTEN (Ye et al., 2014), which is one of the human tumor suppressors. Recent studies have shown that MC-LR can inhibit the expression of PTEN in melanoma and hepatocellular carcinoma (Li et al., 2017). However, whether the miR-221 participates in the inhibition of PTEN which is induced by MC-LR remains unknown.

The signal transducer and activator of transcription 3 (STAT3) is an important nuclear transcription factor that regulates the migration and invasion of hepatocellular carcinoma (Zhang et al., 2014). PTEN negatively regulates STAT3 activation in HPV-infected papilloma cells (Sun and Steinberg, 2002). Deletion of the PTEN gene by small

Abbreviations: MC-LR, Microcystin-LR; PI3-K/AKT, Phosphatidylinositol 3-kinase/Akt; PP1 and PP2A, Protein phosphatase type 1 and type 2A; qPCR, Quantitative real-time polymerase chain reaction; STAT3, Signal transducer and activator of transcription 3; siRNA, Small interfering RNA; MMP, Metal matrix proteinase

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interfering RNA disinhibit the activity of STAT3 (Ji et al., 2011). Phosphorylation of STAT3 was not expressed in overexpressing PTEN U-87MG cells, which is implanted in rat brains (Moon et al., 2013). Additionally, STAT3 up-regulated the protein expression and transcriptional activity of β-catenin through binding to STAT3-binding sites as the gene promoter of β-catenin in breast cancer (Armanious et al., 2010). β-catenin can translocate to the nucleus where it acts as a transcription factor affecting not only cell survival rate, but also cell motility by regulating its target genes (Ou et al., 2011). Nevertheless, the association of STAT3, β-catenin and the cell motility remains unknown in colon cancer cells after MC-LR exposure.

Here we proved that MC-LR up-regulated the level of miR-221, decreased the expression of PTEN and inhibited the phosphorylation of STAT3. We also demonstrated that MC-LR enhanced carcinoma cell motility by regulating the cross-talk between miR-221/PTEN and STAT3 signal in human colon cancer DLD-1 cells.

2. Materials and methods

2.1. Cell culture and exposure

DLD-1 cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences. Cells were maintained in DMEM (Hyclone, Logan, UT) supplemented with 10% (v/v) FBS (Gibco, Grand Island, NY), 100 units/ml streptomycin and 100 units/ml penicillin (Gibco, Grand Island, NY) in a humidified incubator (5% CO_2, 37 °C). Microcystin-LR with a purity of ≥ 95% (Enzo, New York, NY) was dissolved in methanol (1 μM stock solution) and then added into the medium in 0.1% volume ratio dilution to reach different concentrations (0, 1, 5, 12.5, 25, 50 nM).

2.2. Transfection

STAT3 siRNAs and miR-221 inhibitor were purchased from GenePharma (Shanghai, China) and PTEN expressing plasmid was kindly provided by Dr. Fan Lin (Department of Cell Biology, Nanjing Medical University). PTEN expressing plasmid was conducted from a full length cDNA fragment of the human *Pten* gene (1212 bp) which was amplified via PCR and was cloned into the pLenti CMV Blast DEST vector (#17451, addgene) using EcoR I and BamH I restriction enzymes. All of them and related negative controls were used in transfection with Lipofectamine 2000 (Invitrogen, USA) according to manufacturer's protocol. More details of sequence information were displayed in Table S1. Cells were seeded in six-well plates with a density of 8×10^4 cells per well and transfected with STAT3 siRNAs, or miR-221 inhibitor, or PTEN expressing plasmid and related negative controls for 6 h in Oligofectamine in serum-free Opti-MEM medium (Invitrogen, CA) according to the manufacture's instruction. Then cells were incubated at 37 ℃ in a humidified atmosphere of 5% CO2. 48 h later, cells were collected and used for next research.

2.3. Microarray analysis

Total RNA was extracted from DLD-1 cells using TRIzol (Invitrogen) and miRNeasy mini kit (QIAGEN, Denmark) according to manufacturer's instructions. The RNA quality and quantity were measured by NanoDrop ND-8000 (Thermo Fisher Scientific). RNA was subjected to miRNAs expression analysis using the Affymetrix miRNA microarray (Affymetrix, USA) according to the manufacturer's recommendations. The extraction of raw data was used Affymetrix GeneChip Command Console (version 4.0, Affymetrix) software. Then, data were subjected to normalization background correction and summarization using Expression Console (version1.3.1, Affymetrix) software with the standard Robust Multi-array Average (RMA) algorithm.

2.4. Real time qPCR analysis

Total RNA was extracted with Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol from DLD-1 cells. Complimentary DNA was synthesized from 10 μl of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The reaction mixture was as follows: 10 μl of reverse transcriptase reaction mixture containing 2 μl of 10 × RT Buffer, 0.8 μl of 25 × dNTP Mix (100 mM), 2 μl of 10 × RT Random Primers, 1 μl of MultiScribe™ Reverse Transcriptase, and 4.2 μl of RNase-freed H_2O. The mixture was incubated at 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 min.

The expression levels of mRNA was assessed with RT-qPCR using Power SYBR® Green (Applied Biosystems, USA) by an Applied Biosystems 7500 Sequence Detection system. Each reaction was with a final volume of 20 μl including 10 μl of SYBR Green PCR Master Mix, plus 1 μl of each primer and 2 μl of template DNA and 6 μl H_2O. The reaction was repeated for 40 cycles. The expression levels of miRNA were defined based on the threshold cycle (Ct), and the relative expression levels were calculated by using the $2^{-\Delta\Delta Ct}$ method, with the expression levels of U6 small nuclear RNA as reference. Primers of miR-221 and U6 are listed in Table S1.

2.5. Western blot

Total protein from DLD-1 cells was extracted by RIPA lysis buffer (Sunshine Biotechnology, Nanjing, China) containing 1 mM PMSF (Beyotime, Shanghai, China) and protease inhibitor cocktail (Biotool, Houston, TX). Protein concentration was detected by BCA kit (Beyotime, Shanghai, China). Then 30 μg/well protein was separated in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The PVDF membranes were immunoblotted orderly with primary antibodies-against STAT3 (1:1000, #8768), phospho-STAT3 (1:1000, #9145), PTEN (1:1000, #9188) and secondary antibody (1:2000, #7074). All antibodies used in western blot were purchased from Cell Signal Technology (Danvers, MA). The protein bands were visualized with an enhanced chemiluminescence kit (Thermo Scientific, Rockford, IL) and analyzed by a scanning densitometer (TANON 5200, Shanghai, China) with molecular analysis software (BioSens Gel Imaging System, BIOTOP). GAPDH (1:1000, #5174) was used as an internal control.

2.6. Immunofluorescence staining

Cells grown on coverslips were fixed with 4% paraformaldehyde for 20 min at room temperature and were permeabilized with 0.3% Triton X-100 in PBS for 15 min to measure the expression of β-catenin. Then coverslips were blocked with blocking buffer consisting of 4% bovine serum albumin in PBS for 30 min at room temperature and were incubated with primary β-catenin monoclonal antibodies (1:100, #8480 T, CST) overnight at 4 ℃, followed by incubation with the secondary anti-rabbit fluorescein isothiocyanate-labeled antibody (1:200, ZSGB-BIO, China) at room temperature and dark environment for 1 h. Finally, cells were stained with 5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen) for 5 min, and stained cells were captured using confocal microscopy (Carl Zeiss, Germany).

2.7. Migration assay

Briefly, cell migration assay were performed on 24-wells plates with 8.0 μm pore transwell inserts (Costar, Cambridge, MA, USA). DLD-1 cells were incubated with or without MC-LR (50 nM)/STAT3 siRNA, MC-LR (50 nM)/miR221 inhibitor, MC-LR (50 nM)/PTEN expressing plasmid and related negative controls for 48 h at 37 °C in a humidified atmosphere of 5% CO_2. DLD-1 cells, which had migrated to the