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Inhibitory Effect on the Hepatitis B Cells through the Regulation of miR-122-MAP3K2 signal pathway

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Abstract: The aim of this study was to investigate the inhibitory effect of regulation of miR-122-MAP3K2 signal pathway on the hepatitis B cells. We detected the content of MAP3K2 from patients with HBV blood serum samples and analyzed the correlation between content of MAP3K2 and copies of HBV-DNA. Wound healing and Transwell assays were used to detect the function of cells from control group (wild type) and observer group (overexpresses miR-122). Secretion levels of HBsAg and MAP3K2 in the supernatant and level of MAP3K2 in cells were detected by ELISA and western blot, respectively. The results showed that there was a positive correlation between the copies of HBV-DNA and MAP3K2 in serum. In the assays involving detection of the number of HBV-DNA copies, the supernatant levels of HBsAg and MAP3K2, and the level of MAP3K2 in the cells, the rate of increase of these indicators significantly slowed as culture time. In conclusion, overexpression of miR-122 could inhibit the migration of hepatoblastoma cells; however, following transfection with miR-122, DNA synthesis and the secretion of HBsAg were inhibited. Overexpression of miR-122 can also downregulate MAP3K2. Consequently, we concluded that regulating the miR-122-MAP3K2 signaling pathway exerts an inhibitory effect in hepatitis B cells.

Key words: Hepatitis B cells, miR-122, inhibit, signaling pathway.

INTRODUCTION

Cirrhosis is an independent predictor of liver cancer development in patients with chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections (Bal and Onlen 2018, Cheng et al. 2018). It is necessary to evaluate the extent of fibrosis in daily clinical practice when diagnosing patients who suffer from chronic liver diseases. Exosomes, a type of extracellular vesicles, originate from the inner endosomal membrane, which include microRNAs (miRs) and small noncoding RNA molecules (19–25 nucleotides) that regulate gene expression on a post-translational level (Alivernini et al. 2017, Shyamasundar et al. 2018). There are a number of endogenous miRs that serve indispensable roles in the translation of the genetic code and expression of

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proteins. It has been previously reported that there is a correlation between HBV infection and certain miRs, and therefore this group of miRs should receive more attention due to their potential for acting as biomarkers for disease diagnosis (Rohan et al. 2018, Burmi et al. 2019, Ruiz Esparza-Garrido et al. 2017). miR-122 is an miR abundant in the healthy liver, which plays a role in stimulating the translation of HBV and HCV RNA. A relationship between miR-122 and HBV infection has been previously reported, and, thus, an increasing number of studies now focus on its value as a candidate therapeutic biomarker for the assessment of liver cancer treatment (Bandopadhyay et al. 2016, Wojcik et al. 2016, Hoffmann et al. 2012).

MATERIALS AND METHODS

PARTICIPANTS

A total of 27 patients with HBV admitted to the First Affiliated Hospital of Guangxi University of Chinese Medicine, arranged from May 2017 to May 2018. The blood samples were collected and then, HBV-DNA copies and content of MAP3K2 in the samples were detected by RT-PCR and ELISA. The participants were informed and signed written consent forms before the procedure. Moreover, the Medical Ethics Committee of Guangxi University of Chinese Medicine approved the study and the experiments have already been in accord with the ethical standards established by the institution. In order to protect the patients' privacy, the patients' names were replaced by digital locks.

EXPRESSION OF miR-122 WAS ENHANCED IN HEPG2.2.15 CELLS BY TRANSFECTION TO OBTAIN THE HEPG2.2.15+Hsa-miR-122 CELLS (THE OBSERVER GROUP)

Packaging of the Hsa-miR-122 lentivirus was conducted according to published protocols, and purification of the infectious viral particles was achieved through ultrafiltration centrifugation. For cellular transduction of the Hsa-miR-122 lentivirus, 1×10^5 cells/well were seeded into 6-well plates. Cells were transduced with the Hsa-miR-122 lentiviral construct. After 12 hours, cells were screened with puromycin. Infection efficiency was subsequently evaluated using light and fluorescent microscopy, and, at 12 hours after infection, cells were harvested and used for subsequent experiments.

GROUPS

HepG2.2.15 cells in the logarithmic growth phase were divided into two groups, depending on the different treatment they received when cultured in DMEM. The observer group included HepG2.2.15 cells cultured in DMEM supplemented with 10% FBS and Hsa-miR-122, while the control group HepG2.2.15 cells were treated with an equal volume of PBS to Hsa-miR-122.

TRANSWELL ASSAYS

Transwell chambers (Corning, USA) with an 8-mm pore polycarbonate filter were irradiated with ultraviolet light to ensure the elimination of microorganisms. For the migration assays, HepG2.2.15 cells in the logarithmic phase were collected and washed ≥3 times with PBS. Cells were then digested in trypsin. After centrifugation and collection, the cells were resuspended in DMEM at a density of $1x10^6$ cells/ml, and 100 µl was seeded into each of the upper chambers. A volume of 600 µl of DMEM (containing 10% FBS) was added into the lower chambers. Twenty-four hours later, the Transwell chambers were stained with crystal violet for 30 min and washed with ddH_2O ≥3 times to ensure that nonspecific staining was removed. Subsequently, the chambers were placed in a fume hood and dried naturally. The number of cells that had migrated to the lower chamber in 10 randomly selected bright fields was counted manually.

WOUND HEALING ASSAY

Logarithmic growth phase HepG2.2.15 cells were used in this assay and the proliferative capacity of different groups of cells was detected. Cells were seeded onto 6-well plates and allowed to proliferate until they reached 90% confluence. A single line of constant width was scraped in the center of each well. Afterwards, HepG2.2.15 cells were washed with PBS 3 or more times to remove detached cells. Then, the cells in the different groups were cultured in DMEM containing 10% FBS and different additives. Cells at the wound edge were observed and images were captured using an OLMBS microscope at a magnification of x200 for each well at specific time points (0, 12, 24, and 48 h).

DETECTION OF HBV-DNA, miR-122 RNA COPIES AND SECRETION LEVELS OF HBSAG

Logarithmic growth phase HepG2.2.15 cells were resuspended in DMEM, containing 10% FBS, at a concentration of 1×10^5 cells/ml and 1 ml was added to each cell flask. When the cells adhered to the flasks, the original medium was discarded and washed out, and 5 ml of fresh DMEM containing different additives was added (Hsa-miR-122 in the observer group and an equal volume PBS in the control group). 500 µl of culture supernatant was collected at specific time points (24, 72 and 120 h, respectively) and used for the detection of HBV-DNA copies and the secretion levels of HBsAg. And all the cells in the cell flask were collected at specific time points, using for miR-122 detection by RT-PCR. HBV-DNA and miR-122 RNA copies were detected with PCR Thermal Cycler (ABI7300, USA) and primer sequences provided by the company (Huada Gene, China). The secretion levels of HbsAg were detected in a method of immunoluminescent (ADC-CLIA-400, Autobio, China).

LEVEL OF MAP3K2 DETECTION BY WESTERN BLOT

We collected the HepG2.2.15 cells from control group and observer group at specific time points, isolated in the lymphocyte separation medium by density-gradient centrifugation with radioimmunoprecipitation assay buffer (San Diego, CA, USA). Separated the protein, and transferred to the PVDF membranes. And then after the membranes were blocked with milk, plumbed the membranes with relevant primary antibody and secondary antibodies respectively.

STATISTICAL ANALYSIS

The data are expressed as the mean ± SD. Differences between the means of two groups were analyzed by the Student's *t*-test; the contents of different substances were analyzed by one-way ANOVA and the *F*-test, the correlation between MAP3K2 content and HBV-DNA copies were analyzed by linear correlation analysis. Statistical analyses were performed using SPSS 16.0 software, and *P*-values of <0.05 were considered statistically significant.

RESULTS

A total of 27 patients with HBV enrolled in the study, arranged from May 2017 to May 2018, we analysed HBV-DNA copies and content of MAP3K2 in the blood serum samples and the result was showed in the Figure 1 and Table I, there was a positive correlation between the copies of HBV-DNA and MAP3K2 in serum (correlation index $R^2=0.251$).

A new cell line overexpressing miR-122 was generated based on HepG2.2.15, and this cell line was named "HepG2.2.15+Hsa-miR-122". As shown in Figure 2, HepG2.2.15 cells were cultured with the Hsa-miR-122 viral solution for ~12 hours, and the infection efficiency of 90% was evaluated using light and fluorescent microscopy. The results indicated that we successfully constructed a cell

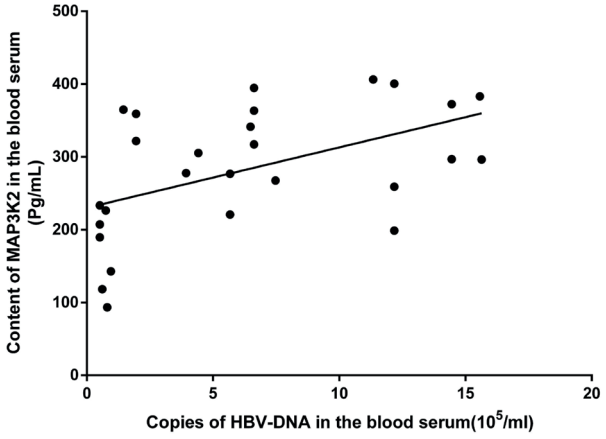

Figure 1 - Correlation analysis between content of MAP3K2 and copies of HBV-DNA. The figure shows that there was a positive correlation between the copies of HBV-DNA and MAP3K2 in serum (correlation index R^2=0.251).

line (HepG2.2.15+Hsa-miR-122) to complete the subsequent experiments.

In the cell function tests, enhanced expression could effectively inhibit directional cell migration in the HepG2.2.15+Hsa-miR-122 cells (Figure 3a and b). And in the Transwell assay, enhanced expression of miR-122 could inhibit the migration of cells as well and a mobility of 60% was recorded in the observer group while 80% in the control group (Figure 3c and d).

For assays involving the detection of HBV-DNA copies, HBsAg secretion levels and MAP3K2 content, we noted that as the time increased, the growth rate of these indicators in the cell supernatant significantly increased. Furthermore, in the observer group, the secretion rate of these indicators was slower than in the control group (Table II, Figure 4a, b and c).

The expression levels of MAP3K2 were detected by western blot. Compared with control group, the result of WB as shown in the Figure 5, and the protein levels of MAP3K2 in the HepG2.2.15 cells samples from observer group were dncreased ($P < 0.001$).

TABLE I
Copies of HBV-DNA and content of MAP3K2 in the serum from 27 cases.

Sample NO.	Content of MAP3K2(pg/ml)	Copies of HBV-DNA ($\times 10^5$)
1	259.14	12.18
2	296.76	14.45
3	220.83	5.67
4	277.79	3.93
5	305.25	4.41
6	317.10	6.62
7	233.37	0.51
8	118.31	0.61
9	267.61	7.47
10	400.50	12.18
11	296.43	15.64
12	383.21	15.57
13	359.15	1.95
14	406.26	11.34
15	394.74	6.62
16	207.27	0.51
17	142.86	0.96
18	226.46	0.75
19	198.79	12.18
20	372.36	14.45
21	276.77	5.67
22	321.86	1.95
23	364.91	1.45
24	363.55	6.62
25	189.58	0.51
26	93.37	0.81
27	341.52	6.48

DISCUSSION

Liver cancer, especially mediated by HBV, accounts for the first place of prevalence in the global statistic of patients with liver cancer. HBV x protein (HBx) (Yu et al. 2016, Liang et al. 2016, Peng et al. 2014), plays an important role in the induction of HBV-mediated liver cancer and facilitates the deterioration of the condition through a series of signaling pathways or factors involved in cell cycle progression, such as inactivation of P53 (Li et al. 2013, Sendi 2012), Ras-Raf-MAP kinase pathway and inhibition of DNA repair. miR-122, is the most

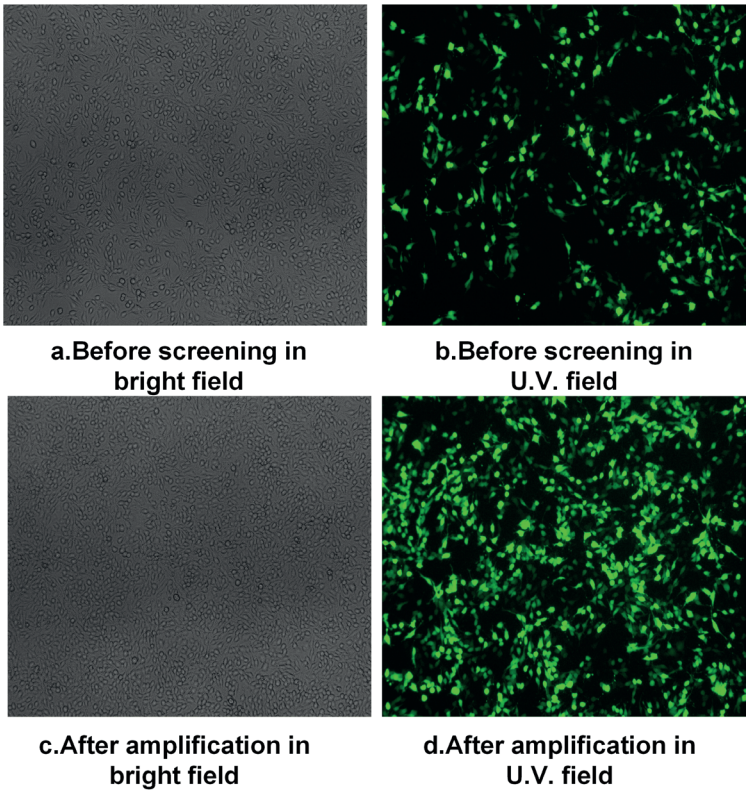

Figure 2 - Panels **a** and **b** show the HepG2.2.15 cells cultured with Hsa-miR-122 viral solution before screening. **c** and **d** show that the number of HepG2.2.15 cells increased after screening with puromycin.

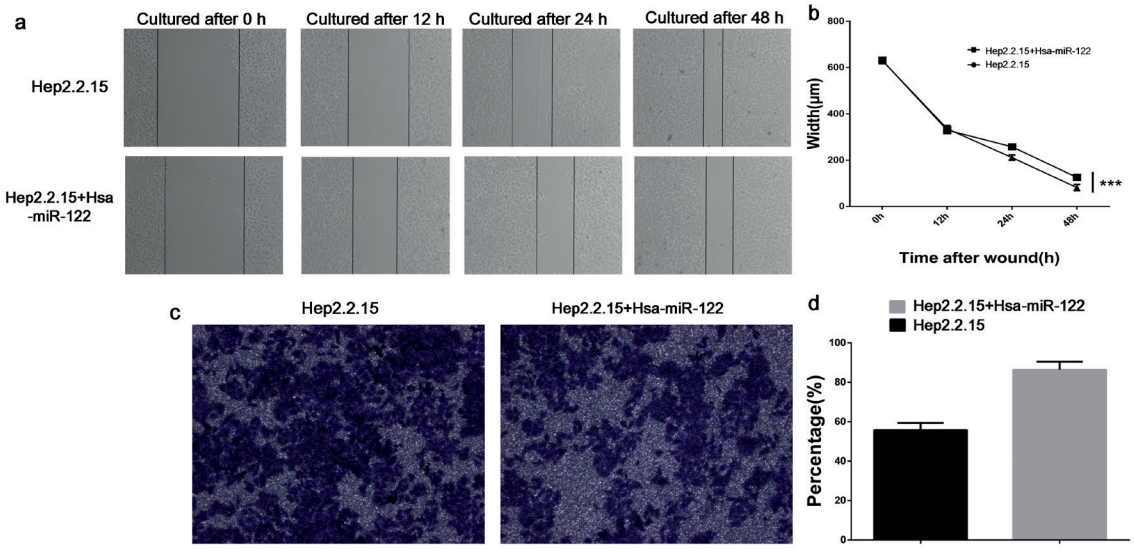

Figure 3 - Cell function test. **a** Wound healing assay. Two groups were cultured for 48 hours, and the result were quantitatively represented using the curve, which indicated that the observer group exhibited the lower degree of healing. **b** Transwell assay. After 24 hours of culture, a cell counter was used to calculate the percentage of cells in each field of view. A mobility of 60% was found in HepG2.2.15 cells treated with Hsa-miR-122.

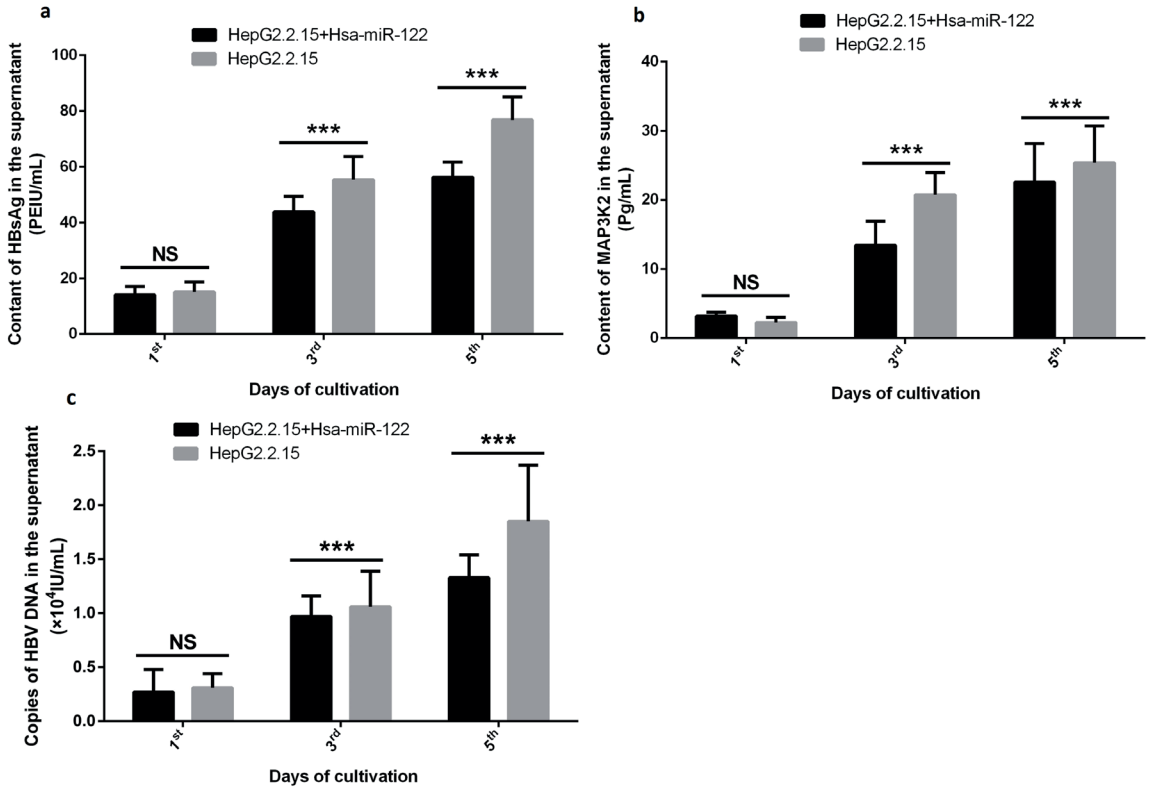

Figure 4 - The number of HBV-DNA copies **a**, levels of HbsAg **b**, and levels of MAP3K2 **c** in the cell culture from different groups at different time points (24 h, 72 h and 120 h) are shown in Figure 4 and Table II. The number of HBV-DNA copies and the levels of HbsAg and MAP3K2 gradually increased in an equal volume of cell supernatant in a time-dependent manner. However, the rate of increase of the three indicators in the observer group was significantly inhibited, compared with in the control group.

TABLE II
Detection of the number of HBV-DNA copies, the levels of HbsAg and MAP3K2 in the cell culture supernatant at different time points.

Time/Groups		HepG2.2.15	HepG2.2.15+Hsa-miR-122	*F*	*P*
HBV-DNA (×10⁴IU/mL)	1st day	0.31±0.13	0.27±0.21	58.70	0.001
	3rd day	1.06±0.33	0.97±0.19		
	5th day	1.85±0.52	1.33±0.21		
HBeAg (PEIU/mL)	1st day	15.15±3.47	14.07±2.99	30.04	0.001
	3rd day	55.35±8.39	43.89±5.43		
	5th day	76.83±8.26	56.28±5.44		
MAP3K2 (pg/mL)	1st day	2.26±0.745	3.18±0.557	6.052	0.01
	3rd day	20.73±3.256	13.44±3.494		
	5th day	25.37±5.363	22.56±5.581		

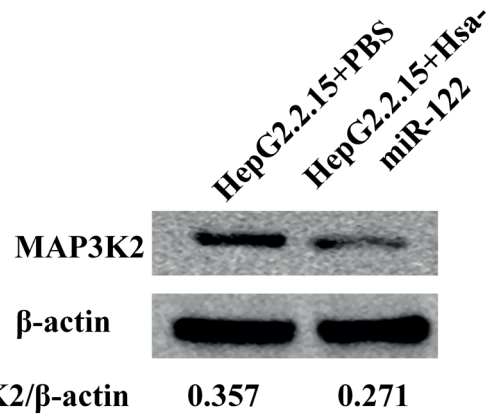

Figure 5 - Western Blot assay. The WB analysis of MAP3K2 expression in the blood samples from control group were significantly higher than the samples from observer group.

abundant miR in liver tissues of healthy people, however, its expression is significantly lowered in liver cancer, especially in the HBV-mediated liver cancer (Qiu et al. 2010, Fan et al. 2011). Previous studies demonstrated that miR-122 was downregulated by HBx, via the reduction of Gld2 (Germ Line Development 2) (Peng et al. 2014, Dai et al. 2017, Yan et al. 2017, Qiao et al. 2017). In our study, we found that in the patients with HBV, there was a positive correlation between the copies of HBV-DNA and MAP3K2 in serum, and increasing the content of miR-122 in the cells can achieve a certain degree of inhibition in the wound healing assay and a significant inhibitory effect on the biological function (migration and secretion of HbsAg) of HepG2.2.15 cells. Some researchers have shown that this signaling pathway is related to MAP3K2, and it is consistent with what we have found in our study.

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AUTHOR CONTRIBUTIONS

Conception and design of the study: Yinghui Lin and Lei Yang; Acquisition of data: Songlin Chen and Aiping Pan; Analysis and interpretation of data: Lei Yang and Siliang Duan, Mingfen Li; Drafting the article: Songlin Chen, Jingjing Huang, Ping Li and Mingfen Li; Revising it critically for important intellectual content: Jingjing Huang and Xingxin Gao, Xiaoqi Huang; Final approval of the version to be submitted: Yinghui Lin and Lei Yang; Songlin Chen, Lei Yang, Aiping Pan contributed equal to this article.

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