MiR-203 mejora la inmunidad tumoral después de la hepatectomía parcial en ratas cirróticas con carcinoma hepatocelular

MiR-203 Enhances the Tumor Immunity after Partial Hepatectomy in Cirrhotic Rats with Hepatocellular Carcinoma

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Resumen
La hepatectomía parcial sigue siendo el tratamiento terapéutico más común para el carcinoma hepatocelular (CHC), la alta recurrencia y metástasis de CHC es la causa principal de muerte después de la resección hepática. La inmunidad tumoral desempeña un papel fundamental en la aparición y el desarrollo de tumores malignos. Sin embargo, aún no está claro cómo se puede abolir el crecimiento aumentado y la metástasis del CHC residual inducido por la regeneration hepática promovida después de la resección hepática, y la inmunidad tumoral se puede mejorar o no. En este estudio, se verá un modelo de rata con cirrosis hepática y CHC difuso, se administrará adenovirus miR-203 recombinante para inducir la sobreexpresión hepática de miR-203 y se seguirá una hepatocarcinoma parcial (PH) del 30%. Se investigó el efecto de miR-203 sobre la inmunidad tumoral, la función hepática y la alfafetoproteína (AFP) después de la HP. Descubrimos que la sobreexpresión de miR-203 puede mejorar la expresión de los linfocitos T CD8 + (CD8) y los linfocitos T reguladores CD4 + CD25 + reguladores (Tregs) en los tejidos de para-carcinoma después de HP, lo que mejora la inmunidad tumoral de las ratas modelo con HCC Además, si miR-203 puede ser una terapia clínica específica en CHC o no, merece una investigación adicional.

Palabras clave: carcinoma hepatocelular, modelo de ratas, sobreexpresión de MiR-203, hepatectomía parcial, inmunidad tumoral

Abstract
Partial hepatectomy is still the most commonly therapeutic treatment for hepatocellular carcinoma (HCC), the high recurrence and metastasis of HCC is the main cause of death after liver resection. Tumor immunity plays a pivotal role in the occurrence and development of malignant tumor. However, how the augmented growth and metastasis of residual HCC induced by the promoted liver regeneration following liver resection can be abolished remains unclear, and if tumor immunity can be enhanced or not was also unknown to us. In this study, a rat model with liver cirrhosis and diffuse HCC was established, recombinant miR-203 adenovirus was administered to induce hepatic miR-203 overexpression and 30% partial hepatectomy (PH) followed. The effect of miR-203 on tumor immunity, liver function and Alpha-fetoprotein (AFP) following PH was investigated. We found that the overexpression of miR-203 can improve the expression of CD8+ T lymphocytes (CD8) and down-regulating CD4+CD25+ regulatory T lymphocytes (Tregs) in para-carcinoma tissues after PH, thus enhance the tumor immunity of model rats with HCC. Moreover, whether miR-203 can be a specific clinical therapy used in HCC or not is worthy of further research.

Key words: Hepatocellular carcinoma, Model rats, MiR-203 overexpression, Partial hepatectomy, Tumor immunity

1.Introduction
Hepatocellular carcinoma (HCC) is the second leading cause of global cancer deaths and the incidence is rising[1], advances in medical therapy have resulted in improved survival for many common malignancies, but not HCC[2]. The lack of curative treatments and consequent pessimistic survival rates make elucidating HCC biology and translating this into effective treatments a priority. There is a good evidence that HCC stimulates an immune response, making immunotherapy a potential option in which the host’s immune system being stimulated to attack the cancer. However, most clinical trials of tumor immunotherapy have been turned out disappointing. Partial hepatectomy is still the most effective treatment to cure HCC, however, it can promote the
growth of HCC in the residual liver at the same time, the possible mechanisms include the changes of inflammatory cytokines, the changes of tumor growth microenvironment and the reduction of immunity, etc[3].

Tumor immunity plays a pivotal role in the occurrence and development of malignant tumor, the main effector cells including CD3+ T lymphocytes (CD3), CD8, CD4+ T lymphocytes (CD4) and Tregs, etc. CD8 are among the most valuable one, researches on them are also more sufficient[4], the most common function approach is cytotoxic effect[5, 6], and the decreasing of CD8 can accelerate the progression of HCC[7]. Moreover, the balance between T lymphocytes subsets is important in maintaining stable immune environment, the CD4/CD8 ratio is one of the evaluation indexes of immunity, it is suggested that when the ratio is reduced, it generally means a descent of immunity[8].

Tregs has been firstly reported in 1995[9], Yang’s study suggested that Tregs in patients with HCC may control CD8’s activity and contribute to the progression of HCC[7]. Mathai also proved that the higher expression of Tregs in HCC patients was associated with higher progression, lower differentiation, and even poorer prognosis[10].

MiR-203 is a newly discovered microRNA and involved in the occurrence and development of various malignant tumors as a tumor suppressor or carcinogen[11, 12]. Murakami reported the abnormal expression of miRNAs in the tumor and adjacent non-tumorous tissues of patients with HCC[13], through the systematic comparison and analysis of miRNAs profiles expression in patients with HBV, HCV, cirrhosis and HCC, he found that the abnormal expression of miRNAs was significantly correlated with the occurrence and development of HCC. Chen[14] reported the relationship between the expression of miR-203 in HCC patients and the recurrence and survival after liver transplantation, the results showed that the expression of miR-203 in patients with recurrence(n=16) was significantly lower than that in patients without recurrence(n=50)(P=0.003). And patients with higher expression of miR-203 tended to have a better five-year survival (P = 0.014) and lower recurrence rate (P = 0.003).

Although previous study has indicated that miR-203 can influence the occurrence and development of HCC by regulating the relevant target genes, but whether it can influence the tumor immunity of patients with HCC by regulating the expression of relevant T lymphocytes in para-cacinoma tissues or not is still unknown. In our previous study for a rat model of liver cirrhosis accompanied by diffuse HCC, we found that overexpression of miR-203 could inhibit the augmented proliferation and metastasis of HCC residual in the promoted regenerating liver after partial hepatectomy[15]. This study was designed to investigate the effect of miR-203 on tumor immunity of HCC after PH.

2. Materials and Methods

2.1. Animal model

A rat model with liver cirrhosis and diffuse HCC was induced as the same protocol described in our prior published study[15], the venous blood was harvested for liver function analysis and AFP detection, the liver tissues were fixed with 4% paraformaldehyde for hematoxylin and eosin (H&E) stain. The details were repeated described in Fig.1.

![Fig. 1](image-url)  
**Fig. 1.** Confirmation of rats model with cirrhosis and diffuse HCC. (a) Hepatic cirrhosis and diffuse HCC confirmed by naked eye, HE from representative cases were also shown, too (HE original magnification, 2009). (b) Interferences of adenovirus injection and PH were performed. (1) Rats of the miR-203+PH group were injected with recombinant miR-203 overexpression adenovirus through the penis vein. (2) HCC nodules were observed
after abdominal incision. (3) PH was performed in the rats of the miR-203+PH, miR-NC+PH, and PH groups. (4) Liver specimen of left lobe resected by PH. (c) blood samples of abdominal aorta were harvested.

Following the confirmation of the liver cirrhosis and diffuse HCC (Fig.1a), 10 days after the final administration of diethylnitrosamine (DEN), rats with high activity were selected, and randomly divided into four groups: a miR-203 transfection plus PH group (miR-203+PH, n = 12), a miR-negative control transfection plus PH group (miR-NC+PH, n = 12), a PH only group (n = 12), and a sham group (n = 16), the specific interferences of different group was describe in our prior published study[15]. All rats were killed on the 15th day after PH or laparotomy, blood samples of abdominal aorta were harvested (Fig.1c), partial tumor tissues from liver were fixed with 4% paraformaldehyde (PFA) for H&E and immunohistochemistry (IHC) stain, the other tumor tissues from liver were harvested for qRT-PCR assay, and partial non-tumor cirrhotic tissues were fixed with 4% (PFA) for IHC stain[15].

2.2 Analysis of liver function, AFP and HE
Liver function and AFP were tested by Fully Automatic Biochemical Analyzer (BS-120; Mindray, Shenzhen, China) and ELISA (SEA153Ra 96T assay kit; Cloud-Clone, Shanghai, China), respectively. Pathological examination for hepatic cirrhosis and diffuse HCC was performed by H&E stain. Pathological grades of HCC were evaluated by Edmondson-Steiner stage[16].

2.3 Detection of hepatic adenovirus transfection
Two pathologists assessed the slides without knowledge of subgroups of rats and were blinded to each other’s evaluation. The expression of miR-203 in hepatic on the postoperative day (POD) 0 and POD 15 was analyzed with qRT-PCR. Liver tissue of POD 0 were embedded in frozen paraffin blocks and cut into 4-μm sections. DAPI (Sigma), at the concentration of 1 μg/mL, was included for the identification of nuclei, and GFP was examined with a fluorescence microscope (ECLIPSE 80i; Nikon, Sendai, Japan).

2.4 QRT-PCR analysis for miR-203 expression
Total RNA was isolated from the liver tissues by TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), and the first strand cDNA was synthesized using a mirRNA 1st-Strand cDNA Synthesis Kit (Shanghai Newgene Biosciences, Shanghai, China). qRT-PCR analyses for mRNA of miR-203 and β-actin were conducted with Power SYBR Master Mix (Invitrogen). MiRNA levels were quantified using ViiA7 (Applied Biosystems, Foster City, CA, USA) and the 2-ΔΔct relative quantification method.

2.5 Western blot for detection of protein expression
Protein expression levels were assessed by western blot. In brief, tissues (20–50 mg) were lysed in lysis buffer containing 1 PBS, 1% NP-40, 0.1% SDS, 5 mmol/L EDTA, 0.5% sodium deoxycholate and 1 mmol/L of sodium orthovanadate and protease inhibitors. The target protein was then probed using antibodies (1:1000, Santa Cruz, CA, USA), and rabbit anti-tubulin-alpha (1:500, Santa Cruz) antibody was used to detect tubulin-alpha, which served as an internal control.

2.6 Detection of hepatic T lymphocytes expression by IHC
The tissue samples were cut into 4-μm sections. After antigen retrieval, the sections were incubated overnight with the first antibody (CD3, Ab16669; 1:100; CD4, MCA55GA, AbD.1:200; CD8, MCA48GA, AbD.1:100; CD25, MCA273GA, AbD). A standard two-step immunoperoxidase-labeled protocol with goat anti-rat HRP (EnVision, DAKO, Glostrup, Denmark) was applied stringently on all slides. IHC analysis results was evaluated by immunohistochemistry score (IHS), IHS=A × B (A: positive cell number; B: grading of color intensity of positive cells, 0 [negative], 1 [weak positive], 2 [positive], 3 [strong positive]). Two pathologists assessed the slides without knowledge of subgroups of rats and were blinded to each other’s evaluation.

2.7 Statistical analysis
The difference in numeric variables between groups were analyzed using one-way analysis of variance, χ2 or Fisher’s exact tests were performed to determine the ratio of Edmondson–Steiner stage. Statistical analyses and graphing were conducted using SPSS 20.0 (IBM, Chicago, IL, USA), and GraphPad Prism 7 software (GraphPad Company, La Jolla, CA, USA). P < 0.05 was considered a significant difference.

3. Results

3.1 The confirmation of rats model with cirrhosis and diffuse HCC
As was reported in our previous study[15], H&E stain showed every rat was induced successfully with cirrhosis and diffuse HCC with most of them are Edmondson I and II (89.6%) (Fig.1a). At the same time, tumor
stage was also judged, the ratio of stage I + II and stage III + IV were calculated to detect the pathological progression. The ratio of stage III + IV in the PH group rats was significantly higher than that of the sham group, and there was no significant difference between the miR-203+PH group and the miR-NC+PH group. The AFP of peripheral blood was significantly higher than normal (167.713 ± 35.629 ng/mL), liver function of model rats was still in the compensatory stage.

3.2. Hepatic miR-203 overexpression was induced by miR-203 adenovirus transfection

Expression of GFP of liver tissues was detected by fluorescence microscope at POD 0, GFP expression in the miR-203+PH group rats was significantly higher than that of the other three groups. qRT-PCR was performed to detect the relative hepatic levels of miR-203 at POD 0 and POD 15. Hepatic miR-203 expression in the miR-203+PH group rats was significantly higher than that of the other three groups, and this effect lasted to POD 15[15].

3.3. MiR-203 overexpression influences the expression of relevant T lymphocytes in para-carcinoma tissues of model rats after PH

Expressions of CD3, CD4, CD8 and Tregs in para-carcinoma tissues were detected by IHC. Expression of CD3 and CD8 have no significant difference between different groups, Tregs expressed in PH group was significantly higher than that in gram group and significantly lower in MiR-203+PH than in miR-NC+PH group, CD4 expression in miR-203+PH group rats were significantly higher than that in miR-NC+PH group, while the difference was not significant between other groups. The ratio of CD4/CD8 was significantly higher in miR-203+PH group than that in miR-NC+PH group, and this ratio in PH group was significantly lower compared to sham group. Meanwhile, in miR-203+PH group, CD8 expression was negatively correlated with Tregs (Fig.2, Fig.3a,b).

Fig.2. IHC was performed to detect CD3, CD4, CD8, and Tregs. Images from representative cases are shown (IHC, ×400).

Fig.3. MiR-203 overexpression influence the expression of relevant T lymphocytes in para-carcinoma tissues of model rats after PH. (a) CD3 and CD8 have no significant difference between different groups, CD4 were significantly higher in the miR-203+PH compared with miR-NC+PH group, the expression of Tregs was significantly lower in miR-203+PH group compared with miR-203-NC+PH group; (b) The ratio of CD4/CD8 was
significantly higher in rats of miR-203+PH group than that in miR-NC+PH group, and the ratio in PH group was significantly lower compared to sham group.

4. Discussion

Rats are the most widely used animal models of medicine trials which can be induced with HCC within 20 weeks by using DEN[17], in our study, rat livers were subject to a sequential process of toxic injury, cirrhosis and tumorogenesis. This pathological process is similar to the carcinogenesis of human HCC induced by hepatitis B virus infection. H&E stain showed every rat was induced successfully with cirrhosis and diffuse HCC with most of them are Edmondson I and II (89.6%) according to the Edmondson-Steiner classification. At the same time, tumor stage was also judged, the ratio of stage I + II and stage III + IV were calculated to detect the pathological progression. The ratio of stage III + IV in the PH group rats was significantly higher than that of the sham group, and there was no significant difference between the miR-203+PH group and the miR-NC+PH group.

CD3 is the codifferentiation antigen of mature T lymphocytes, they can represent total T lymphocytes and partly reflects the functional status of tumor immunity. CD4 mainly involved in the recognition of T cell receptor (TCR) antigens and the transmission of T lymphocytes activation signal, it is suggested that Tregs inhibits the activation and proliferation of CD4 through cell-cell contacts, thereby inhibiting tumor immunity and accelerating the growth of tumor. CD4 is a critical element in optimal activation of CD8 and in the maintenance of immunological memory[18], they can participate in tumor immunity not only by activating cytotoxic T lymphocyte (CTL) or CD8, but also other related cells or directly killing target cells[19, 20].

In our study, IHC results showed that the expression of CD3 and CD8 had no significant difference between different groups, moreover, CD4 expression in miR-203+PH group rats were significantly higher than that in miR-NC+PH group(P<0.0001), while the difference was not significant between other groups(P >0.05), which pointed out that the overexpression of miR-203 can improve the expression of CD4 in para-carcinoma tissues of model rats after PH. In consideration of the result that the expression of CD8 had no significant difference between different groups, and there was no significant correlation between CD4 and CD8 in miR-203+PH and miR-203-NC+PH group (r=-0.445 , P=0.27;r=-0.281, P=0.54), so the classic pathway that from CD4 to CD8 to target cells effect may not applicable in this study, and if CD4 can enhance the tumor immunity of model rats after PH by other means still needs further research.

Tregs mainly involved in the maintaining of immunological homeostasis and regulating autoimmune status, its specific immunosuppression function may inhibit tumor immunity and cause malignant tumor to evade immune surveillance. Mathai[10] confirmed that the overexpression of Tregs in patients with HCC were associated with higher progression and lower differentiation of tumors, and possibly indicated a poorer prognosis. It is also found that the expression level of CD3 in HCC is positively correlated with tumor size[21]. Recently years, the relationship between Tregs and HCC has been clarified with the in-depth study of tumor immunology. Pedroza has pointed out that the expression of Tregs has been increased significantly with the progression of HCC, and the elimination of Tregs could inhibit the proliferation of tumor, which suggested that Tregs might induce the immunologic escape of HCC[22]. Previous studies have treated Tregs as immunosuppressive cells[23-25], Sasaki have shown that Tregs can be acted as an independent predictor of the recurrence of HCC, the higher expression of Tregs often indicates a shorter disease-free survival[26]. Kobayashi also noted that patients with lower levels of Tregs in HCC had better overall survival rates than those with higher levels[27]. In our study, the Tregs expressed in PH group was significantly higher than that in gram group (P<0.0001), suggesting that PH can lead to a elevation of Tregs, thereby inhibiting tumor immunity, thus lead to tumor recurrence. At the same time, Tregs expressed in miR-203+PH group was significantly lower than in miR-NC+PH group (P<0.0001), which indicated that the overexpression of miR-203 can reduce the expression of Tregs in para-carcinoma tissues after PH. Then we have detected the correlation between Tregs,CD4 and CD8 expressed in miR-203+PH group, the results showed there was no significant correlation between Tregs and CD4 (r=-0.014,P=0.973), whereas there was a significant negative correlation between Tregs and CD8 (r = 0.808, P = 0.015), which indicated that the overexpression of miR-203 can reduce Tregs in para-carcinoma tissues, and then increase the expression of CD8, and thus enhance the tumor immunity of model rats after PH. In consideration of that CD8 has no significantly difference between different groups, so further studies are needed to confirm whether it was due to the insufficient injection volume of miR-203, or unrepeatd injection.

Liver function examination showed that the peripheral blood was collected at the 14th day after PH in order to reduce the influence of other interventions, because of the powerful regeneration ability of rats liver, the results that liver function and AFP between different groups of model rats after PH had no significant different was easy to understand, and the result also showed that the Wistar rats have the characteristics of strong tolerance and high adaptability, moreover, it is an effective method to improve the sensitivity and reliability of the experiment by optimize experiment design.
5. Conclusion

By summing up our data we can safely draw the conclusion that the protocol to induce model rats with liver cirrhosis and diffuse HCC in Wistar rats by CCl4 subcutaneous injection with DEN drinking water was simple and reliable, and the overexpression of miR-203 can improve the expression of CD8 and down-regulating Tregs after PH, thus enhance the tumor immunity of model rats with HCC. Moreover, whether miR-203 can be a specific vaccine used in HCC clinical treatment or not is a problem worthy of further research.

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