Mechanism of dact2 gene inhibiting the occurrence and development of liver fibrosis

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ARTICLE INFO

Original paper
Article history:
Received: August 15, 2021
Accepted: November 26, 2021
Published: December 30, 2021

Keywords:
Liver Fibrosis; Dact2 Gene; Chronic Liver Disease; Development Mechanism

ABSTRACT

There are few reports about the relationship between dact2 and liver fibrosis. The inhibitory mechanism of dact2 in liver fibrosis is not clear, we need to further explore it. In this study has been shown that the dact2 gene can inhibit liver fibrosis. In this experiment, we used lentivirus as a vector to construct a lentivirus vector carrying the dact2 gene and packaged dact2 recombinant lentivirus and its control vector. HSC-T6 infected cells were observed. The effect of dact2 gene expression was activates Wnt3a HSC-T6 cells. Immunoblot was used to detect α-SMA expression, TGF-β 1, Smad3, Smad7, β-Catenin and CyclinD1. The expression of MMP-2 and TIMP-1 was detected by real-time PCR. At the same time, dact2 recombinant lentivirus was injected into the tail vein. Carbon tetrachloride was used to establish the liver fibrosis model. After 7 weeks of modeling, the staining was used to observe the pathological changes of liver tissue, hydroxyproline was used to analyze the changes of collagen content in liver tissue, the expression of the protein was observed by immunohistochemistry, and the expression of fibrosis-related genes was detected by real-time PCR. Results showed that the dact2 gene expression could inhibit the activation of HSC-T6 cells and reduce the expression of TGF-β 1. The percentage of Smad3, β-Catenin and cyclinD1 protein was 50.02%, 46.73%, 47.58% and 37.50% respectively (P < 0.05).

DOI: http://dx.doi.org/10.14715/cmb/2021.67.6.5

Introduction

The effective treatment of chronic liver diseases such as posthepatitic cirrhosis and portal hypertension depends on the blocking of liver fibrosis. Liver fibrosis is a reversible disease, but there is still a lack of effective treatment (1-2) in clinical practice. Therefore, it is very important to study and explore new therapeutic methods of liver fibrosis (3-5). A variety of pathogenic factors can induce liver fibrosis, such as chronic viral infection, chronic alcoholism, chemical poisoning or drug poisoning, autoimmune injury and so on (6). Early liver fibrosis can be reversed by external factors (7-8). We are still lack of effective anti-fibrosis treatment methods and cannot be widely used in clinical, so exploring new treatment strategies is still the focus of liver research (9-10).

Liver fibrosis can be caused by a variety of pathogenic factors, including alcohol abuse, drug poisoning, virus infection and so on. At present, a large number of studies have shown that the formation of liver fibrosis is mainly due to the deposition of a large number of ECM proteins in the liver. HSCs are believed to participate in this process (11-13). Hepatic stellate cell is a kind of special perihepatic cell, also known as lipid storage cell. It is located in the perihepatic space of the liver, usually in a static state (14). HSCs were activated and their phenotypes changed from static to active (15-16). Through the contraction of cells, the intrasinusoidal pressure of liver is increased, resulting in portal hypertension (17-18). Activated HSCs are the main source of ECM, and all kinds of fibroblasts take HSCs as the final target cell (19). Therefore, the molecular mechanism of HSCs activation has been the goal of anti-fibrosis research (20). Pay attention to the basic research of liver fibrosis, further explore the molecular mechanism and regulatory points to promote the occurrence and development of fibrosis and the activation of hepatic stellate cells, in order to find new therapeutic targets to effectively reverse the process of

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fibrosis, restore liver function and improve the prognosis of patients with chronic liver disease.

This paper mainly deals with the mechanism of the dact2 gene inhibiting the development of liver fibrosis. Therefore, the method of control experiment is adopted. The results showed that the dact2 gene can inhibit the activation of hepatic stellate cells by reducing the deposition of ECM. Meanwhile, TGF-β1, Smad3, β-Catenin and CyclinD1 decreased by 50.02%, 46.73%, 47.58% and 37.50%, respectively. Gene transfer can be achieved by viral and non-viral vectors (21). Its highly effective and stable expression characteristics make it widely used in clinical experimental research.

Materials and methods

Material Science

46 male SD rats weighing 190-210 g were purchased from the animal experimental center. All mice were raised in the laboratory according to the SPF environment and the regulations on animal feeding management.

Experimental Method

Animal models and groups: 46 SD rats were randomly divided into a normal group (10 rats), liver fibrosis model group (12 rats), dact group (12 rats), empty body group (12 rats). The suspension of lentivirus dact2 and lentivirus EGFP were diluted with 1 x pbs and injected into rats of the dact2 group and empty vector group respectively. A rat model of hepatic fibrosis was established. In the normal group, 0.2ml/100g saline was injected subcutaneously twice a week for 7 weeks. During the whole experiment, one animal died in the model group at the 2nd, 4th, 5th and 6th week, one in the dact2 group at the 6th week, one in the empty body group at the 3rd week and one at the 4th week.

Tissue sample collection: remove the residual blood of liver tissue, and stop the lavage when the color of the liver turns gray and the lavage fluid is clear. The right lobe of the liver near the portal vein of the rat was taken after dissection. Determination of hydroxyproline in liver tissue (alkaline hydrolysis method): weigh accurately 40 mg of tissue wet weight, put it into a centrifuge tube, take it out after water bath for a few seconds, shake it up. Add 1.0mlph-a solution into each tube, and stir evenly. At this point, the solution turns red. Use a 100 μ l micropipette to suck pH adjustment B scheme, and carefully add pH adjustment B solution to each pipe drop by drop. After each drop is added, stir well. When the color of the indicator in liquid turns yellow-green, the pH value is about 6.2-6.9. Add 10ml of double distilled water and stir evenly. Take 2ml of diluted hydrolysate, add 10mg of active carbon, centrifuge at 3400rpm for 10min, and carefully suck 1ml of supernatant for detection. After adding the sample, stir evenly, take a water bath at 50 ℃ for 13min, cool with tap water, and centrifuge at 3400rpm for 10min. Take the supernatant and test the absorbance value of each tube at 500nm. Statistical analysis: all the values in this experiment were expressed by means of mean ± standard deviation. The results were analyzed by SPSS17.0 software. Independent sample t test was used for comparison between the two groups. Single-factor analysis of variance was used for comparison between multiple groups. P < 0.05 was statistically significant.

HE Staining

24 hours before the detection, the liver tissue was taken out from the - 70 ℃ refrigerator and put into the -10 ℃ refrigerator, which was convenient to synchronize with the temperature of the frozen microtome. The frozen tissue mass and embedment agent decreased, the slice thickness was 5 μ m, fixed in reverse desquamation. About 100 μ LD API quickly dropped to slide, and PBS buffered 2 times 3 minutes after nuclear staining, 3 minutes each time. 3 minutes after washing with 0.4% eosin staining water, the sealant after the neutral tree was dehydrated and transparent, observed and photographed under an inverted microscope.

Immunohistochemical Staining

The paraffin section was dewaxed in the oven at 60 ℃, washed with 1 × PBS buffer for 3 times, each time for 3 minutes. The slices were repaired by microwave with EDTA buffer solution. The slices were cooled for 5min after boiling and cooled naturally after boiling. The slices were washed twice with 1 × PBS buffer solution for 4min each time. The repaired sections were incubated in 2% hydrogen peroxide solution at room temperature for 10 minutes to block the action of endogenous peroxidase. Wash 1 × PBS buffer twice for 5 minutes each time, and seal it with
5% BSA for 20 minutes after drying. Each part was diluted with 50 μ l of the main antibody, washed 3 times at 4 °C overnight, and buffed for 3 minutes with 1 × PBS each time. Each part was incubated at 3 °C for 40 minutes, washed twice for 1 × PBS buffer for 3 minutes, added 40-90 μ l of fresh minjillian to solve each part, observed the color development under the inverted microscope, after the completion of color development, washed with clear water, then stained with hematoxylin, divided into 1% hydrochloric acid alcohol (a few seconds), returned to ammonia water blue, washed with clear water. After 60%, 75%, 85% dehydration, anhydrous ethanol and transparency, the neutral resin was sealed and observed under the inverted microscope. PBS buffer solution (ph7.3): na2hpo41.34g, NaCl 7g, kcl0.1g, kh2po40.14g are all dissolved in 700ml of double-distilled water, adjust the pH value to 7.3 with 4mol / l hcl, and then set the volume to 1000ml, sterilize with high-pressure steam, and store at room temperature for standby. RNA extraction and PCR related reagent preparation: 0.1% DEPC water: measure 1ml of diethylpyrocarbonate, add about 800ml of triple distilled water, fully mix, and store at 4°C; 75% ethanol: anhydrous ethanol and transparency, the gel containing the target DNA was prepared by 1% agarose gel electrophoresis. The reaction conditions were 120V, 30min. The electrophoresis results were observed under ultraviolet light, and the gel containing the target DNA band was inserted into the aseptic centrifuge tube. The rubber block was weighed by electronic balance, and the volume of the rubber block was calculated according to 1 mg = 1 μ L. three times the volume of sol solution PN centrifuge tube was added. After mixing, put into a 50 °C water bath, soak in a metal water bath for 10 minutes, heat and melt the rubber block. During this time, turn the centrifuge tube up and down slightly to ensure that the rubber block is completely dissolved. Pre balance of adsorption column: put the adsorption column into the header, add 500 μ LBL to maintain the balance, centrifuge for 1 minute at 15000 rpm, and discard the waste liquid. Add 600 μ LPW adsorption column for cleaning, centrifuge at 15000 rpm for 1 minute, and discard waste liquid. Add 600 μ LPW to wash the adsorption column again, centrifuge it for 1 minute at 15000 rpm, discard the waste liquid and dry it naturally at room temperature. Put it in a centrifuge tube, centrifuge at 15000 rpm for 2 minutes, and then collect the DNA solution.

**Real-Time PCR Detection**

After primer design, 1 μ LDT and 1.0 μ g total RNA were added to the PCR reaction tube, and then the ribonuclease water was added to 12 μ l total RNA. After mixing, centrifugation, cooling on ice for 1 minute at 65°C, 5 minutes, and annealing, the oligodT and template were added immediately. Then add 5 × rtbuffer4 μ L, rnaseinhibitor1 μ L, dntpmix2 μ L, m-mlvreversetranscriptase1 μ l to the reaction solution, mix gently, and briefly centrifuge. RT reaction conditions: 42°C, 1H, 70°C, 5min to inactivate the RT enzyme. The final product is cDNA, which is stored in a refrigerator at - 80°C. RNA extraction: sample processing: cell collection: wash the culture plate cells with 1 × PBS once, add Trizol to dissolve the cells, and gently blow them up for use; tissue collection: off each group of liver tissue blocks, put them into precooled 1.5mlep tube, wash them with 1 × PBS once, grind them with electric grinding rod ice, centrifugate them at 4°C 1000rcf for 5min; take the sediment, add 1mltrizol every 50-100mg of tissue, and gently blow them up for use; The above homogenate was placed at room temperature for 5min to fully decompose the nucleic acid-protein complex; 0.2ml chloroform was added to 1mltrizol, stirred upside down slightly for about 30 times, and placed at room temperature for 5min; 12000rcf was centrifuged at 4°C for 15min. Alcohol precipitation; repeat the previous steps; dry at room temperature for 10 minutes, add 15-20 μ 10.1% DEPC water, the residual ethanol volatilizes completely, and gently attack the dissolved RNA; concentration determination: take the solution of more than 2 μ l RNA, use nd2000 analyzer to determine the concentration and purity of RNA, and twist the remaining RNA, or store it at - 80°C in a short time. When extracting the total RNA, add 0.5ml Trizol into each hole, blow it out carefully with a pipette, and transfer it to another new 1.5mlep tube after the cell is completely cracked and drawn into silk. Add 100μl chloroform to each tube, shake it up and down, mix well, and then at room temperature for 15 minutes. At 4°C,
12000 revolutions / centrifugation for 15 min. carefully aspirate 200 μl of the upper aqueous phase from each tube to another new 1.5 ml EP tube. Add 150 μl isopropanol, mix well, and place on ice for 10 minutes. Centrifuge the supernatant and dry it completely.

### Results and discussion

#### Detection of Dact2 Expression in Liver Tissue

As shown in Figure 1, dact2 recombinant lentivirus dact2 is injected into the rat tail vein. The frozen section shows that the dact2 recombinant lentivirus infection group is mainly located in the portal area, but not in the normal group and model group, which indicates that dact2 is successfully expressed in the liver.

![Figure 1](image1.png)

**Figure 1.** Detection of Dact2 expression in liver tissue.

#### HE Staining of Liver Tissue

As shown in Figure 2, the staining of liver tissue showed that: model group B and empty carrier group D, hepatocyte swelling, deformity and fat, and a large number of inflammatory cells infiltrated in portal area, and fibrous tissue proliferation zone extended to the lobule of the liver; while dact2 group C still had some degree of steatosis, but inflammatory substances and fibrous proliferation in the portal area of liver were significantly reduced.

![Figure 2](image2.png)

**Figure 2.** HE staining of liver tissue.

#### Determination of Hydroxyproline in Liver Tissue

According to the statistical analysis of data, as shown in Figure 3 and Table 1, the expression of hydroxyproline in each group: model group (340.10 ± 12.95 μg / g) and normal group (90.43 ± 7.49 μg / g) significantly increased (P < 0.05), dact2 group (140.43 ± 6.10 μG / g) and model group significantly decreased (P < 0.05); there was no significant difference between model group and empty body group (P > 0.05).

<table>
<thead>
<tr>
<th>Group</th>
<th>Normal group</th>
<th>Model group</th>
<th>Gact2 group</th>
<th>Empty body group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Content of hydroxyproline in liver tissue (μg/g)</td>
<td>90</td>
<td>340</td>
<td>140</td>
<td>320</td>
</tr>
</tbody>
</table>

![Table 1](image3.png)

**Table 1.** Determination of hydroxyproline in liver tissue.

#### Real-Time PCR Detection of Liver Tissue

According to the statistical analysis of data, as shown in Figure 4 and Table 2, the expression of TGF - β 1, smad3smad7, β - Catenin and CyclinD1 mRNA were detected by real-time PCR 7 weeks later, the model was established in mice infected with dact2 recombinant lentivirus. The results showed that the expression of TGF - β 1 and Smad3 mRNA increased by 1.94 and 1.52 times, respectively, compared with the normal group. The expression level of dact2 in the intervention group was 1.56 times higher than that in the model group (P < 0.05). There was no significant difference between the model group and the empty body group (P > 0.05). In addition, the expression level of β - Catenin and CyclinD1 mRNA in the model group were 1.98 times and 1.49 times higher than that in the normal group, respectively, while the expression level of β - Catenin and CyclinD1 mRNA dact2 intervention group were 47.58% and 37.50% lower than that in the model group, respectively (P <
There was no significant difference between the model group and the empty body group (P > 0.05).

<table>
<thead>
<tr>
<th>Classification</th>
<th>Normal group</th>
<th>Model group</th>
<th>Gact2 group</th>
<th>Empty body group</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td>0.9</td>
<td>2.7</td>
<td>1.3</td>
<td>2.4</td>
</tr>
<tr>
<td>Smad3</td>
<td>0.9</td>
<td>2.2</td>
<td>1.2</td>
<td>2.5</td>
</tr>
<tr>
<td>Smad7</td>
<td>0.9</td>
<td>0.3</td>
<td>0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>β-catenin</td>
<td>0.9</td>
<td>2.9</td>
<td>1.2</td>
<td>2.6</td>
</tr>
<tr>
<td>CyclinD1</td>
<td>0.9</td>
<td>2.3</td>
<td>1.4</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Figure 4. Real-time PCR detection of liver tissue.

Damaged hepatocytes and endothelial cells can recruit a variety of inflammatory cells to maintain the homeostasis of the liver by secreting a variety of cytokines, chemokines and other chemical media, as well as regulating the acute inflammatory response and damage repair response. However, if the liver injury continues, inflammatory response and tissue repair cycle alternate, HSCs in the liver will be activated continuously, resulting in an imbalance of extracellular matrix synthesis and catabolism, and fibroid lesions. At the cellular and molecular level, a network system (22-23) was constructed with HSCs activation and transformation regulation as the core, and each factor interacts and restricts with each other to jointly affect the process of liver fibrosis. At present, the treatment strategies of fibrosis mainly include: clearing away the main pathogenic factors; reducing the inflammatory reaction, inducing the apoptosis of activated HSCs; increasing the degradation of extracellular matrix, promoting the reversion of fibrosis (24-27). In this experiment, the staining showed the massive degeneration and necrosis of liver cells, a large number of collagen fibers deposition and the formation of the fibrous septum in the model group, which confirmed that the liver fibrosis model was successful, while the proliferation of damaged liver cells and fibrous tissue dact2 gene expression group was significantly lower than that of the model group, indicating that dact2 expression can reduce liver toxicity. It is generally believed that the content of hydroxyproline in liver tissue can reflect the degree of collagen deposition in the liver.

In this experiment, the staining showed the large degeneration and necrosis of hepatocytes, the deposition of a large number of collagen fibers and the formation of the fibrous septum in the liver tissue of the model group, which confirmed the success of the liver fibrosis model, and the proliferation of damaged hepatocytes and fibrous tissue dact2 gene expression group was significantly lower than that of the model group, indicating that dact2 expression can reduce the hepatotoxicity of aramid. It is generally believed that the content of hydroxyproline in liver tissue can reflect the degree of collagen deposition in the liver. The results showed that the content of hydroxyproline in dact 2 group was significantly lower than that in the model group. These results indicate that the expression of dact2 in the liver can reduce the deposition of ECM.

In theory, the structure and function of liver fibrosis can be restored by removing the pathogenic factors of liver fibrosis, alleviating the inflammatory reaction and carrying out effective anti-fibrosis treatment. Pathogenesis of liver fibrosis: a variety of liver damage factors can cause liver damage and inflammation, and the body can repair the damaged liver to maintain a stable balance. Therefore, excessive ECM formation or reduction of ECM degradation for any reason may lead to ECM over deposition and fibrosis. In the process of fibrosis, many cells in the liver interact with each other, forming a huge cross-cell regulatory network, which affects the occurrence and development of the disease. At present, according to the different functions and functions of different cells in the process of liver fibrosis, it can be roughly divided into three categories: effector cells, guide cells and regulatory cells. Effector cells are cells that directly lead to ECM overproduction and abnormal deposition in the process of fibrosis. HSCs are mainly used as lipid storage cells to participate in vitamin A metabolism, express peroxisome proliferative activated receptors, and maintain low proliferative activity and collagen
synthesis ability. Activated HSCs can promote the development of liver fibrosis through the following aspects: transforming into MFBs with self proliferation ability, migrating and accumulating in the damaged part of liver tissue, producing a large number of ECM mainly composed of collagen; abnormal immune regulation function affects the maturation and function of immune cells by changing a series of chemokine tables, or directly interacting with immune cells entering the liver, Further promote the development of liver fibrosis. Guide cells are cells that provide signals to promote liver fibrosis and guide the activation of hepatic stem cells, including hepatocytes, sinusoidal endothelial cells, etc. Liver parenchymal cells are often damaged in nonalcoholic steatohepatitis and virus infection. The latter can trigger inflammation and fibrosis in the liver. Active oxygen clusters, hedgehog ligands, nucleotides and other regulatory molecules are involved in this process. N-fly is a damage-related molecular model released by damaged hepatocytes, which directly or indirectly promotes the activation and fibrosis of hepatic stellate cells.

Acknowledgements
Key R &D Project Fund of Jiangxi Province, Experimental Study on the Prevention and Treatment of Liver Fibrosis by Tumor Suppressor Gene Dact2, (No. 20161BBG70012).

Interest conflict
None.

References


