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NTCP Change in Rats of Hilar Cholangiocarcinoma and Therapeutic Significance

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Abstract

Background: The study aims to detect the expression of Na^+/taurocholate cotransporter polypeptide in hilar cholangiocarcinoma of rat model, to provide a new therapeutic target for gene therapy of hilar cholangiocarcinoma. **Methods:** 60 male Wistar rats (weighing 190 ± 8 g) were randomly divided into 3 groups (experimental group, control group, and sham operation group; 20 rats in each group). The 3 groups were fed with standard diet. The QBC939 cell suspension of cholangiocarcinoma was injected into the hilar bile duct in the experimental group with a micro syringe. The control group was injected with normal saline, and the sham operation group was not injected with any drugs. Comprehensive behavior score and Basso Beattie Bresnahan were used to evaluate the mental state and exercise of rats every day. At 5 weeks, one rat in the experimental group was killed, and the changes in hilar bile duct were recorded. The procedure was repeated at one and half months. After one and half months, hilar cholangiocarcinoma only occurred in the experimental group. Pathological examination confirmed the formation of tumor, and hilar bile duct tissues were taken from the 3 groups. Na^+/taurocholate cotransporter polypeptide expression in hilar bile duct was detected by real-time polymerase chain reaction and immunohistochemistry. **Results:** After 2 weeks, the rats in experimental group ate less, and their weight was significantly reduced compared with the other 2 groups. One and half months later, hilar cholangiocarcinoma was detected in 16 rats in the experimental group. The levels of alanine aminotransferase and aspartate transaminase in the experimental group were higher than those in the other 2 groups. The ratio of Na^+/taurocholate cotransporter polypeptide/GAPDH mRNA in hilar cholangiocarcinoma, control group, and sham operation group was significantly different. Under the light microscope, Na^+/taurocholate cotransporter polypeptide protein reacted with anti-Na^+/taurocholate cotransporter polypeptide antibody and showed granular expression. Every pathological section included 4800 cells. 3823 positive cells were in the experimental group, 1765 positive cells were in the control group, and 1823 positive cells were in the sham operation group. **Conclusions:** Na^+/taurocholate cotransporter polypeptide expression in hilar cholangiocarcinoma of rats was significantly higher than normal hilar bile duct tissues, suggesting that drugs targeting Na^+/taurocholate cotransporter polypeptide may be a new strategy for the treatment of hilar cholangiocarcinoma.

Keywords

NTCP, hilar cholangiocarcinoma

Abbreviations

BSEP, bile salt export pump; FXR, Farnesylate X receptor; HBV, hepatitis B virus; HDV, hepatitis D virus; NTCP, Na^+/taurocholate cotransporter polypeptide; RT-PCR, real-time polymerase chain reaction.

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Background

Malignant tumors may occur in all organs of the digestive system. Cholangiocarcinoma is only one of them, according to the location of tumor, cholangiocarcinoma can be divided into 3 types: upper cholangiocarcinoma (hilar cholangiocarcinoma), middle cholangiocarcinoma, and lower cholangiocarcinoma. In cholangiocarcinoma,

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hilar cholangiocarcinoma has a special location, it is closer to hepatic artery and portal vein, though its incidence rate is not high, but its malignancy is very high. According to statistical data, the death toll of hilar cholangiocarcinoma has increased year by year in recent years. Because patients with hilar cholangiocarcinoma have no obvious symptoms in the early stage, when obstructive jaundice and liver function damage appear, it has reached the middle or late stage, and its treatment methods are limited.[1–3] Surgical resection is the first choice for patients, but the number of patients who can perform it are extremely limited. According to the bismuth Corlett classification, only type I and type II hilar cholangiocarcinoma meet the requirements of radical resection, but type III and type IV hilar cholangiocarcinoma often can not be completely resected, some patients can be palliative surgery, but the 5-year survival rate is low, some patients can not be operated because of tumor invasion, metastasis, cardiopulmonary insufficiency, and severe liver function damage. Alternative therapies include chemotherapy, radiotherapy, immunotherapy,[4] microwave ablation, radionuclide therapy, etc. However, after clinical practice, they cannot significantly improve the survival rate. Therefore, there is an urgent need for new treatments.

Gene therapy is a new method in tumor treatment in recent years, which has been used in the treatment of some cancers, but the scope of application is not extensive.[5,6] We know from the existing statistical data that the combination of gene therapy and other treatment methods improves the survival rate of patients, improves the quality of life, and has great potential. However, few genes have been found in hilar cholangiocarcinoma. Therefore, we are looking for genes related to the pathogenesis of hilar cholangiocarcinoma as therapeutic targets. Farnesylate X receptor (FXR) is related to bile acid metabolism, its gene and protein expression plays an important role in the occurrence and development of colon, breast, and liver tumors. Insook *et al* have found that mice without FXR gene developed hepatocellular carcinoma or cholangiocarcinoma on the basis of liver cirrhosis. Na^+/taurocholate cotransporter polypeptide (NTCP) is the target gene of FXR,[7–9] it is usually expressed on the surface of hepatocytes, and its function is to reabsorb bile acids. It is an important part of the enterohepatic circulation of bile acids, NTCP mediates the reabsorption of about 80% bile acids from small intestine into hepatocytes, and these bile acids are secreted into bile again to form enterohepatic circulation of bile acids. In previous experiments, we found that FXR and NTCP showed an opposite trend in the hyperlipidemic rat model, the expression of FXR in the hilar cholangiocarcinoma rat model was lower than that in the normal bile duct tissue, because the inhibitor of NTCP had been found, so which made us study the expression of NTCP in hilar cholangiocarcinoma and whether its role in hilar cholangiocarcinoma was enhanced or decreased, Whether NTCP inhibitors have effect on it in tumor tissues. We hope to find a new target for the treatment of hilar cholangiocarcinoma.

Materials and Methods

Statement

The reporting of this study conforms to ARRIVE 2.0 guidelines.[10] The experimental animals received adequate care during the experiment. Animal welfare guidelines abided by Guide for the Care and Use of Laboratory Animals, Eighth Edition.[11] We made efforts to minimize the number of animals utilized and to decrease their suffering. The animals were euthanized by cervical dislocation. *In vitro*, real-time polymerase chain reaction (RT-PCR) shows the changes in NTCP in QBC939 cholangiocarcinoma cells and normal bile duct cells.

Rats (in Vivo)

Sixty Wistar rats (male, 190 ± 8 g, 6 weeks old) were provided by the Animal experimental center of Southwest Medical University. They were randomly divided into 3 groups (the experimental group, the control group, and the sham operation group, $n = 20$ each). Before the study, the rats were healthy and did not take any drugs.

Experimental Methods

Materials: Microsyringe (HAMILTON), Pentobarbital sodium (Beijing Younikang Biotechnology Co., Ltd), QBC939 human cholangiocarcinoma cell line (Shanghai Tongpai Biotechnology Co., Ltd), Hematoxylin eosin staining solution (Nanchang Yulu Experimental Equipment Co., Ltd), DMEM culture medium(Sigma Inc.), anti-NTCP antibody (Chemicon), ABI 7500 real-time PCR detection system. Frozen tissue sections were prepared using freezing microtome, liquid nitrogen, and OCT embedding agent. The main reagents were dNTP, buffer solution, trizol, chloroform, real-time PCR kits (SR1100). The sense and antisense primers used to detect *NTCP* mRNA were as follows: 5′-GATGGAGGTGCACA ACGTAT-3′ and 5′-CTGTC TCAGTTCAT GGCTCC-3′. The sense and antisense primers used to detect *Gapdh* mRNA were as follows: 5′-GATGGTG GGTATGGGTCAGAA-3′ and 5′-CTAGGAGCCAGGGCA GTAATC-3′. The $2^{-\Delta\Delta Ct}$ method was used to express the data. The diameter of the needle tip of the microsyringe is 40 μm, connected to the 100 μL syringe through a rubber tube.

Establish animal model and test. All the Wistar rats were fed with standard diet. QBC939 human cholangiocarcinoma cells were cultured in DMEM medium at 37 °C and 5% saturated humidity. The cells with poor growth were filtered out and the cells with good growth were retained, after the cells with good growth were prepared into cell suspension with the concentration of 1×106 cells/mL, Wistar rats were inoculated. The experimental group was anesthetized with 1.5% pentobarbital sodium and 0.2 mL/100 g intraperitoneal injection. After disinfection, the abdomen was cut along with the median line of the abdomen. Then the microinjector needle was inserted into the hilar bile duct and 100 μL tumor cell suspension was injected. Press to stop bleeding, close the abdominal layers in turn, and complete the operation. Normal saline was injected into the bile duct in the control group, and nothing was injected into the sham operation group. The mental state, diet, and exercise of the rats were evaluated daily by comprehensive behavior

score and Basso Beattie Bresnahan. At 5 weeks, one rat in the experimental group was killed, and the changes in hilar bile duct were recorded. The procedure was repeated at one and half months. Pathological examination confirmed the formation of tumor, and hilar bile duct tissues were taken from the 3 groups. HE (When the tissues were cut into sections, after conventional baking and dewaxing the sections were stained with hematoxylin for 5 min, washed with water for 10 min, stained with eosin for 3 min, and washed with water. Then the sections were dehydrated, transparent, and sealed) staining showed the tissues of hilar cholangiocarcinoma, hilar bile duct tissues of control group and sham operation group. RT-PCR was used to detect the expression of NTCP mRNA (RNA was extracted from hilar cholangiocarcinoma and normal hilar bile duct), GAPDH served as internal control. Immunohistochemical SP method was used to analyze the expression of NTCP protein. Under the light microscope, NTCP protein reacted with anti-NTCP antibody, they showed granular expression. Every pathological section was randomly divided into 6 regions, and 80 cells were observed in each region. The positive cells >10% were positive, and the positive cells <10% were negative.

Statistical Analysis

Data are presented as the mean ± SD. SPSS22.0 statistical software was used for data analysis. The *t* test was used to judge the differences between 2 groups, The χ^2 test was used to evaluate immunohistochemistry data, and $P < .05$ indicates statistically significance differences.

Results

In vitro through RT-PCR, we found that the *NTCP*/*Gapdh* ratios increased in QBC939 cholangiocarcinoma cells than that in normal bile duct cells. *In vivo* after 2 weeks, the rats in experimental group ate less, and their weight was significantly reduced compared with the other 2 groups (Tables 1 and 2). Three rats in the experimental group died after 6 weeks. There were no fatalities in the other 2 groups. Through pathologic examination, we detected hilar cholangiocarcinoma in 16 rats (80%) in the experimental group after 6 weeks (Figure 1). The changes in liver function in 3 groups are shown in Table 3.

Analysis of NTCP Expression

Through RT-PCR we found that in hilar cholangiocarcinoma, control group, and sham operation group, the *NTCP*/*Gapdh* ratios were 42 ± 2.3, 18 ± 1.5 and 19 ± 1.6, respectively. After 8 cycles, there was a significant statistical difference among the 3 groups in *NTCP*/*Gapdh* ratios (between experimental group and control group, $t = 3.015$, $P < .05$, between experimental group and sham operation group, $t = 2.976$, $P < .05$) (Figure 2).

The *NTCP* protein expression which reacted with the anti-*NTCP* antibody is shown in Figure 3. Every pathological section included 4800 cells; 3823 positive cells (79.6%) were in the experimental group, 1765 positive cells (36.8%) were in the control group, and 1823 positive cells (38%) were in the sham operation group, there was significant statistical difference among the 3 groups ($\chi^2 = 32.36$, $P < .05$, between experimental group and sham operation group.$\chi^2 = 33.29$, $P < .05$, between experimental group and control group.).

Discussion

Digestive system malignant tumors include pancreatic cancer, gastric cancer, colon cancer, primary liver cancer, gallbladder cancer, etc. Hilar cholangiocarcinoma presents one of the most malignant phenotypes. Although in the past few years, diagnosis and treatment models have been developed in basic and clinical research, and we have known that the factors related to hilar cholangiocarcinoma include cholelithiasis, congenital bile duct dilatation, clonorchiasis, and primary sclerosing cholangitis, but the pathogenesis of hilar cholangiocarcinoma, the changes in tumor pathophysiology, the mechanism of tumor metastasis and invasion and the regulatory mechanism of the specific signal transduction pathway related to the malignant phenotype of hilar cholangiocarcinoma are still not fully clear.[12–14] For example, hilar cholangiocarcinoma may be caused by the accumulation and interaction of specific factors, but in these factors which can lead to the occurrence of tumor, which can promote the progress of tumor,

Table 1. Daily Food Intake (g).

Week	Control group (n = 20)	Sham operation group(n = 20)	Experimental group (n = 20)
2	19.25 ± 0.11	19.35 ± 0.12	19.22 ± 0.09
3	23.76 ± 0.15	24.07 ± 0.13	21.65 ± 0.11
4	27.72 ± 0.09[a]	28.03 ± 0.12[b]	19.53 ± 0.08
5	28.93 ± 0.13[c]	29.10 ± 0.15[d]	18.79 ± 0.06

[a]$P < .05$ compared with the experimental group.
[b]$P < .05$ compared with the experimental group.
[c]$P < .05$ compared with the experimental group.
[d]$P < .05$ compared with the experimental group. After 2 weeks, the rats in experimental group ate less, and their weight was significantly reduced compared with the other 2 groups.

Table 2. Body Mass (g).

Week	Control group (n = 20)	Sham operation group (n = 20)	Experimental group (n = 20)
2	208 ± 4.7	207 ± 4.5	209 ± 4.6
3	221 ± 6.3	220 ± 5.8	219 ± 5.9
4	238 ± 6.2[a]	239 ± 6.5[b]	206 ± 5.7
5	249 ± 6.7[c]	250 ± 6.3[d]	197 ± 4.2

[a]$P < .05$ compared with the experimental group.
[b]$P < .05$ compared with the experimental group.
[c]$P < .05$ compared with the experimental group.
[d]$P < .05$ compared with the experimental group. After 2 weeks, the rats in experimental group ate less, and their weight was significantly reduced compared with the other 2 groups.

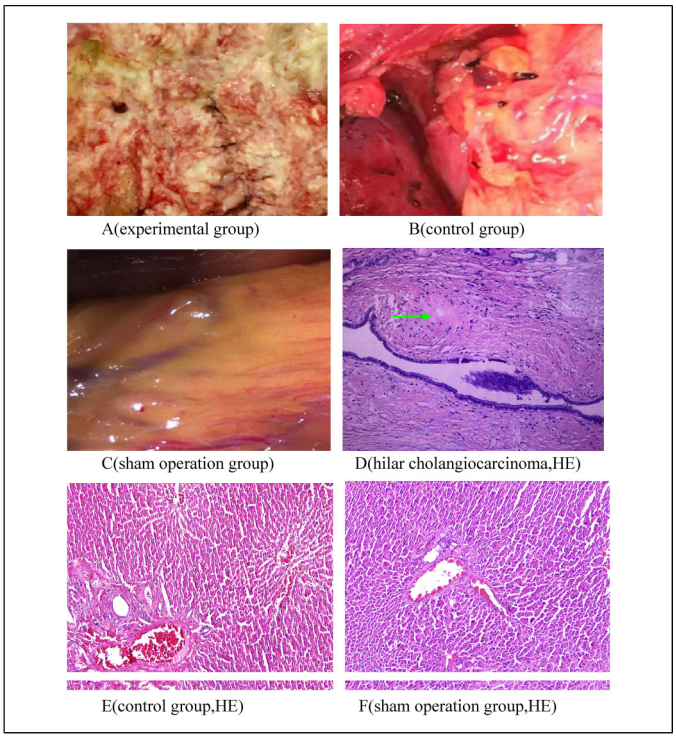

Figure 1. Pathological examination. (A) Hilar cholangiocarcinoma in experimental group. (B) Hilar bile duct in control group. (C) Hilar bile duct in sham operation group. (D) The lobulated masses are indicated by the green arrow in hilar cholangiocarcinoma. HE stain (magnification 100×). (E) Hilar bile duct tissues in control group. HE stain (magnification 100×). (F) Hilar bile duct tissues in sham operation group. HE stain (magnification 100×). One and half months later, hilar cholangiocarcinoma was detected in 16 rats in the experimental group, they are lobulated masses. But in control group and sham operation group only, there were mild inflammation and edema in hilar bile ducts, and a small amount of inflammatory cell infiltration. The tumor cells were multinucleated and the endoplasmic reticulum was swollen. The cells in the control group and the sham operation group were slightly edematous.

Table 3. Changes of Liver Function.

Related indicators	Control group (n = 20)	Sham operation group (n = 20)	Experimental group (n = 20)
(ALT)/(U/L)	82.38 ± 2.69^{a}	83.09 ± 2.71^{b}	157.56 ± 5.28
(AST)/(U/L)	88.07 ± 2.95^{c}	90.17 ± 3.01^{d}	162.15 ± 5.64
(TC)/(mmol/L)	2.57 ± 0.13^{e}	2.56 ± 0.15^{f}	6.48 ± 0.11
(TBA)/(μmol/L)	1.65 ± 0.04^{g}	1.67 ± 0.06^{h}	4.91 ± 0.09
(TBIL)/(μmol/L)	3.87 ± 0.05	3.86 ± 0.04	3.88 ± 0.06
(DBIL)/(μmol/L)	0.74 ± 0.02^{i}	0.76 ± 0.03^{j}	2.01 ± 0.05

Abbreviations: ALT, alanine aminotransferase; AST, aspartate transaminase; TC, total cholesterol; TBA, total bile acids; TBIL, total bilirubin; DBIL, direct bilirubin.
[a]$P < .05$ compared with the experimental group.
[b]$P < .05$ compared with the experimental group.
[c]$P < .05$ compared with the experimental group.
[d]$P < .05$ compared with the experimental group.
[e]$P < .05$ compared with the experimental group.
[f]$P < .05$ compared with the experimental group.
[g]$P < .05$ compared with the experimental group.
[h]$P < .05$ compared with the experimental group.
[i]$P < .05$ compared with the experimental group.
[j]$P < .05$ compared with the experimental group.
The levels of total cholesterol, total bilirubin, direct bilirubin, alanine aminotransferase, and aspartate transaminase, in experimental group were higher compared with the other 2 groups.

which factors can regulate each other, we are not clear, that may explain the lack of effective treatment. Usually, if the tumor is small or limited to a part of the hilar bile duct, it can be completely removed, and the prognosis is good. But if the tumor infiltrates surrounding tissues or metastasizes, surgery is not possible. The effects of interventional therapy, microwave ablation, and immunotherapy are limited. The recurrence and metastasis rate are high, and the 5-year survival rate is low. Therefore, there is an urgent need for more effective diagnosis and treatment. Gene therapy is a new method in recent years and has a good application prospect, although it has been used in the treatment of lung cancer and liver cancer, but it is not widely used in other tomors. Therefore, we are looking for genes related to the pathogenesis of hilar cholangiocarcinoma as therapeutic targets.[15–17]

NTCP usually expressed on the surface of hepatocytes and bile ducts, and its function is to reabsorb bile acids. It is an important part of the enterohepatic circulation of bile acids. Bile acids are first synthesized by hepatocytes, and bile is transported to the intestine by bile salt export pump (BSEP). After bile acids enter the small intestine, about 95% of the conjugated bile acids are reabsorbed through the apical sodium-dependent bile acid transporter, ileal bile acid-binding protein, and terminal sodium-dependent bile acid transporter. NTCP mediates about 80% of bile acids into hepatocytes, and these bile acids are secreted into bile again to form enterohepatic circulation of bile acids.[18–21] Therefore, NTCP plays an important role in bile acid concentration stability. If the expression level of NTCP is abnormal, bile acid reabsorption disorder may occur. When NTCP increases, bile acid reabsorption is excessive, bile will accumulate in the bile duct, components in bile may deposit in the bile duct, leading to cell degeneration, cholangitis, and stone formation.[22–24] The long-term existence of cholangitis and bile duct stone is one of the possible reasons to promote the growth of hilar cholangiocarcinoma. When NTCP decreased, bile acid reabsorption was insufficient, and the enterohepatic circulation of bile acid could not proceed smoothly. Therefore, a better understanding of the expression of NTCP in hilar cholangiocarcinoma plays an important role in exploring new treatment methods.[25–28] When we looked up the data in the early stage of our experiment, we found that there was no significant difference between the NTCP of rats and that of human in terms of both overall and key segments, and the response to drugs was also very similar. In human hilar cholangiocarcinoma, we have found that NTCP is higher than that in normal tissues. Therefore, we chose to inject QBC939 cell suspension into rat bile duct to establish hilar cholangiocarcinoma model and observe the expression of NTCP and its response to blocking agent.[29]

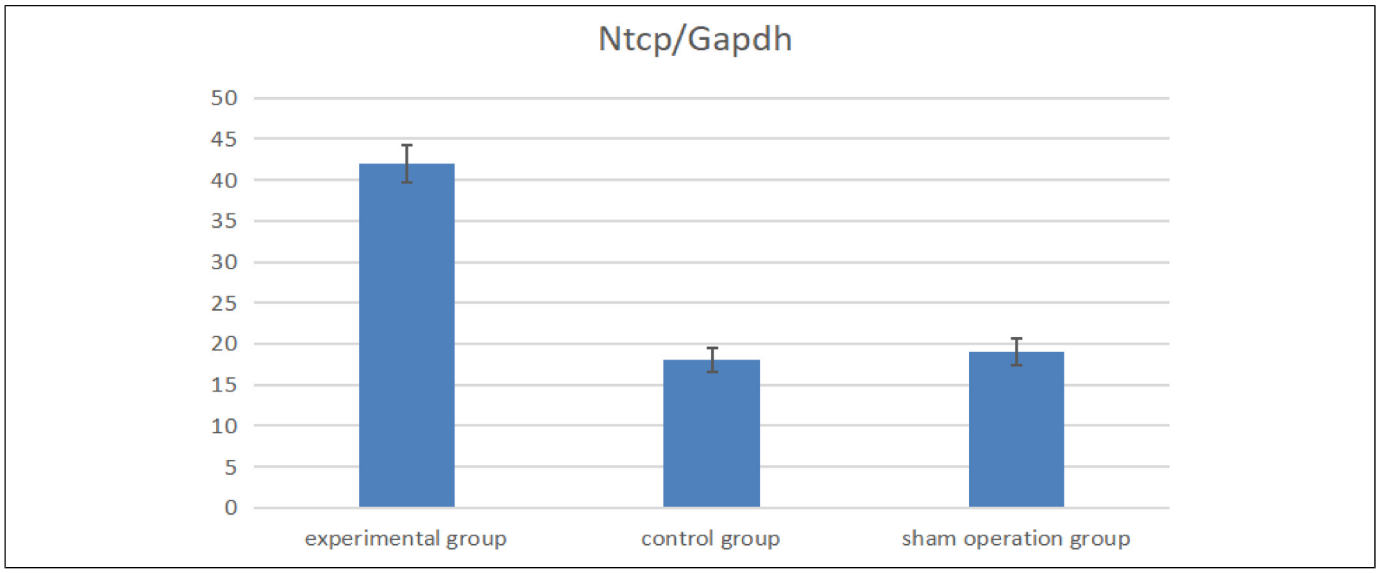

Figure 2. Analysis of *NTCP* mRNA expression by real-time polymerase chain reaction (RT-PCR). Through RT-PCR we found that in hilar cholangiocarcinoma, control group and sham operation group, the *NTCP*/*Gapdh* ratios were 42 ± 2.3, 18 ± 1.5, and 19 ± 1.6, respectively. After 8 cycles, there was a significant statistical difference among the 3 groups (between experimental group and control group, $t = 3.015$, $P < .05$, between experimental group and sham operation group, $t = 2.976$, $P < .05$).

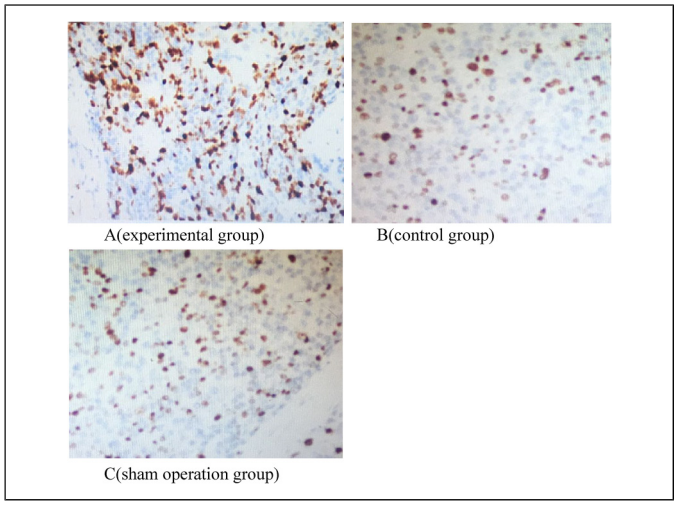

Figure 3. Analysis of *NTCP* expression by immunohistochemical assay. (A) *NTCP* expression in hilar cholangiocarcinoma of experimental group (magnification 200×). (B) *NTCP* expression in normal hilar bile duct of control group (magnification 200×). (C) *NTCP* expression in normal hilar bile duct of sham operation group (magnification 200×). The *NTCP* protein expression that reacted with the anti-*NTCP* antibody are shown in Figure 3. Every pathological section included 4800 cells. 3823 positive cells (79.6%) were in the experimental group, 1765 positive cells (36.8%) were in the control group, and 1823 positive cells (38%) were in the sham operation group, there a was significant statistical difference among the 3 groups ($\chi^2 = 32.36$, $P < .05$, between experimental group and sham operation group. $\chi^2 = 33.29$, $P < .05$, between experimental group and control group).

Previous studies have shown that NTCP is a specific receptor of hepatitis B virus (HBV). In addition to acting as a receptor of HBV infection, it can also participate in the regulation of HBV transcription after infection. The coinfection of hepatitis D virus (HDV) and HBV almost appeared the phenomenon of virus decline after the treatment of NTCP blockers (bulevirtide), Vitro cell experiments have found that the NTCP express in cells of hepatocellular carcinoma, and NTCP blockers can inhibit the proliferation of hepatoma cells. Animal experiments have also found that NTCP increased in liver cancer tissues, and NTCP inhibitors can inhibit tumor growth. We previously found that FXR expressed in liver cancer and cholangiocarcinoma of rats, it decreased in cholangiocarcinoma. Because the tumors of liver and biliary system often affect each other, so we tried to analyze whether the expression of NTCP, the target gene of FXR, has a similar change in the tumors of biliary system of rats. Is NTCP expressed in hilar cholangiocarcinoma? Is the expression increased or decreased? Can NTCP blockers play a therapeutic role? Because hilar cholangiocarcinoma is often difficult to be radical resected in biliary system. Therefore, we designed this experiment to preliminarily understand the expression of NTCP in hilar cholangiocarcinoma, at the same time, because FXR is the regulatory gene of NTCP, so we observed the changes in FXR. In the study through drug injection after 6 weeks, the experimental group successfully established the experimental model of hilar cholangiocarcinoma induced by QBC939. The food intake of rats in the experimental group decreased, and then the body weight decreased. In the detection of liver function, the levels of total cholesterol, total bilirubin, direct bilirubin, alanine aminotransferase, and aspartate aminotransferase in the experimental group were higher than those in the other 2 groups. Simultaneously, muddy stones emerged from the bile ducts of rats in experimental group, the expression of NTCP in hilar cholangiocarcinoma was higher than that in control group and

sham operation group. Therefore, we confirm a problem that if the amount of bile acids in bile duct increases, the expression of NTCP will decrease,[30–32] which will accelerate the secretion of bile acids and reduce the reabsorption of bile acids, so as to maintain the stability of enterohepatic circulation. However, in hilar cholangiocarcinoma, the expression of NTCP greatly increased, and bile acid reabsorption significantly increased. Therefore, cholestasis in the bile duct, the components in the bile may deposite to form stones, causing cell degeneration and inflammation around the bile duct, resulting in the phenomenon of bile duct cell destruction, proliferation, and destruction alternately. The repeated destruction and proliferation of bile duct cells increase the possibility of malignant phenotype cells.

At present, there are few drugs for hilar cholangiocarcinoma, and there are various problems in the treatment process. For example, the scope of their use is very limited. On the one hand, only a small number of tumor tissues with clear pathological classification will respond to drugs. On the other hand, they need a large dose, which has adverse effects on liver and kidney function and digestive system, causing liver and kidney dysfunction and gastrointestinal bleeding, so their tolerance is poor. The drugs in the research and development stage do not directly target the genes related to hilar cholangiocarcinoma. So after the experiment of NTCP expression detection, we are doing another experiment. In preliminary studies, we have found that the NTCP blocker bulevirtide inhibits hilar CCA tumor formation in rats (unpublished findings); however, these studies are still ongoing. The relationship between drugs, NTCP and hilar cholangiocarcinoma, is like the relationship between police, informants, and criminal groups. The police arrest criminal groups through informants, while drugs treat hilar cholangiocarcinoma through NTCP. This study helps us to find a new method for hilar cholangiocarcinoma, but there are limitations at present. Although we have found the changes in NTCP in hilar cholangiocarcinoma, the information obtained in the current study may enhance our understanding of the molecular basis and signal pathway changes in hilar cholangiocarcinoma,[33–35] but the changes in genes related to NTCP need to be further clarified, the effect and influencing factors of drugs on NTCP need to be further observed. In the next 5 years, there will be more research in this field to try new treatments. From the existing experimental results, gene therapy has a good development prospect in this field. And then let us try to find new and more effective treatment strategies from the changes in NTCP.

Conclusion

In this study, we found that NTCP change in rats of hilar cholangiocarcinoma. It increased obviously in hilar cholangiocarcinoma, suggesting that drugs targeting NTCP may be a new strategy for the treatment of hilar cholangiocarcinoma.

Authors' Note

M.Z. and X.X. were responsible for the design; J.W. provided administrative support; M.L. and K.H. did experiments and collected data; M.Z. and J.W. were responsible for manuscript writing. All the authors approved the final manuscript. The study protocol was approved by the Ethics Committee of the affiliated hospital, Southwest Medical University, Luzhou, Sichuan Province, China. Number:2020415 (updated approval number: 2022525).

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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