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ORIGINAL ARTICLES

Treatment of chronic proliferative cholangitis with c-myc shRNA

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Abstract

AIM: To investigate the feasibility and effectiveness of c-myc shRNA in inhibiting the hyperplastic behavior and lithogenic potentiality of chronic proliferative cholangitis (CPC), in order to prevent stone recurrence and biliary restenosis.

METHODS: An animal model of CPC was established by giving intralumenally 0.5 mL of c-myc shRNA. Then, the effects of c-myc shRNA on hyperplastic behavior and lithogenic potentiality of CPC were evaluated by histological observation, immunohistochemistry, real-time PCR and Western blotting for c-myc, proliferating cell nuclear antigen (PCNA), procollagen Ⅲ, mucin 5AC, enzymatic histochemistry for β-glucuronidase, and biochemistry for hydroxyproline in the diseased bile duct.

RESULTS: Treatment with c-myc shRNA efficiently suppressed the hyperplasia of biliary epithelium, submucosal gland, and collagen fiber by inhibiting mRNA and protein expression of c-myc. More importantly, it decreased the lithogenic potentiality of CPC by inhibiting the expression of mucin 5AC and the secretion of endogenous β-glucuronidase. Further investigation indicated that c-myc shRNA-3 had a better inhibitory effect on CPC.

CONCLUSION: Treatment with c-myc shRNA-3 can control CPC and reduce the lithogenic potentiality of CPC.

Key words: Chronic proliferative cholangitis; Hepatolithiasis; Recurrence; C-myc; Prevention

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INTRODUCTION

Hepatolithiasis, a commonly encountered disease in the Asian-Pacific region, is more refractory to surgical treatment than most other benign diseases of the biliary tract[1,2]. Its therapeutic challenges include the difficulty in completely correcting the biliary stenosis and a high rate of stone recurrence, leading to a re-operative rate of 37.1%-74.4%[1-3]. Unfortunately, how to prevent the stone recurrence and biliary restenosis is still a problem to be solved in hepatobiliary surgery. Since hepatectomy cannot eliminate the possibility of stone recurrence, 16% of postoperative patients may develop new stones at other sites[3-5]. Therefore, surgery itself cannot achieve its long-term therapeutic effectiveness on hepatolithiasis[2-7]. In recent years, with a deeper understanding of the pathological changes in hepatolithiasis, the high stone recurrence rate and biliary restenosis rate in hepatolithiasis patents have been found to be related to the residual chronic proliferative cholangitis (CPC) after operation[8-11].

In the past, we paid too much attention to the improvement of surgical skills for treatment of hepatolithiasis, but failed to sufficiently recognize the connection of CPC to the formation of intraheptic calculi, and to pay enough attention to the treatment of residual CPC after removal of stones[3,12-14]. Thus, even though the stone is removed completely and the biliary tract stenosis is corrected, the residual CPC induced by the stone would still exist persistently and

extensively, which would facilitate formation of new stones by producing mucoprotein or by changing the lithogenic pathology and biliary stricture, which leads to cholestasis. Therefore, treatment of CPC after operation might increase its curative effect on hepatolithiasis. Unfortunately, there is no definitely effective therapy for CPC at present[15-17]. Since hepatolithiasis is a chronic proliferative disease, we designed this study to investigate the preliminary effectiveness of c-myc shRNA on hyperplastic behavior and lithogenic potentiality of CPC, expecting to prevent stone recurrence or biliary restenosis by controlling or eradicating CPC[18,19].

MATERIALS AND METHODS

Study design and surgical procedure

A total of 56 Sprague-Dawley rats weighing 220-250 g were randomly divided into six groups. (1) CPC group (n = 10) in which a 5-0 nylon thread was inserted into the common bile duct through the duodenal papilla[20]. (2) Four c-myc shRNA treatment groups (n = 10), in which a nylon thread was used as a guidwire and a 20 G veinous retaining needle was introduced into the common bile duct. Then, a total of 3×10^9 plaque-forming units (pfu) of four kinds of c-myc shRNA (shRNA-1, shRNA-2, shRNA-3, and a negative control sequence provided by Genesil Biotechnology Co. Ltd, Wuhan, China) in a total volume of 0.5 mL mediated by liposome 2000 (Invitrogen, USA) were respectively infused. (3) Sham operation (SO) group (n = 6) in which the common bile duct was dissected only. Transfection efficiency was detected after 48 h. One week later, all the rats were sacrificed with their common bile ducts removed, and fixed in liquid nitrogen and 10% formaldehyde for further tests.

Immunohistochemistry or immunofluorescence staining of c-myc and mucin 5AC

The avidin-biotin-peroxidase complex method was used to detect the expression of c-myc. Briefly, tissue sections were incubated overnight at 4℃ with primary antibody (Zymed Co, USA), followed by incubation with biotinylated second antibody for 1 h at 37℃. Expression of mucin 5AC was detected with immunofluorescence staining. Briefly, cryostat slides were incubated with primary antibodies (Santa Cruz Biotechnology, USA) at 4℃ overnight. After incubation with secondary antibody labeled with fluorescein at 37℃ for 1 h, the expression of mucin 5AC was observed under a fluorescent microscope at once.

Detection of c-myc and mucin 5AC by real time-PCR

Total RNA was extracted from the bile duct wall using Trizol (Gibco, USA). Reverse transcription was performed according to the manufacturer's instructions (Gibco, USA). Real-time analysis was performed on the cycle (Bio-Rad, Germany) using SYBR Green (TaKaRa, Dalian, China). Levels of target gene expression in the tested samples were normalized to the corresponding GAPDH mRNA transcript.

Detection of c-myc, PCNA and procollagen Ⅲ by Western blotting

After protein concentration was determined, 100 μg protein was loaded in each lane and subjected to 8% SDS-PAGE gel electrophoresis, then transferred to nitrocellulose membrane for immunoblotting. The blots were probed with antibodies against c-myc, proliferating cell nuclear antigen (PCNA) and procollagen Ⅲ (dilution 1:1000, Zymed Co, USA) overnight at 4℃. After washed with TBST, the membrane was incubated for 2 h with HRP-conjugated rabbit anti-rat secondary antibody (dilution 1:3000). Immunoreactive bands were visualized with enhanced chemiluminescence and captured on a X-ray film.

Enzymatic histochemical staining of endogenous β-glucuronidase in bile duct wall

The method of Ballantyne was used to perform enzymatic histochemical staining using nephthol-AS-SI-β-D-glucuronide (β-G; Sigma) as the substrate[21]. Briefly, cryostat sections were incubated at 37℃ for 1 h in a pH-4.95 solution containing the β-G substrate and hepatocyte nuclei were counterstained with methyl green for 3 min. Positive expression of endogenous β-G was observed as a rose-red signal in cytoplasm.

Assessment of hydroxyproline content (mg/g of bile duct)

Connective tissue in the blie duct was estimated by quantifying hydroxyproline, an amino acid found primarily in collagen, the principal component of extracellular matrix. The hydroxyproline content was detected as previously described[22].

Statistical analysis

All the data were presented as mean ± SD and analyzed using the SPSS10.0 software. Statistical analysis was conducted using the non-parametric ANOVA to evaluate the variance among more than two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Histopathological examination

Biliary epithelium mucosa in the CPC group was histologically characterized by papillary hyperplasia projection, which led to obstruction of the bile duct lumen. Analogical histological changes were observed in the negative control group. In contrast, proliferative degrees of biliary epithelium, submucosal gland, and collagen fiber in the three c-myc shRNA treatment groups were obviously lower than those in the CPC group, especially in the c-myc shRNA-3 group. More specifically, the degree of fibrous thickening in the diseased bile duct wall was obviously relieved after treatment with c-myc shRNA (Figure 1A-C).

Determination of c-myc and PCNA by immunohistochemistry, RT-PCR and Western blotting

To determine the anti-proliferative effect of c-myc

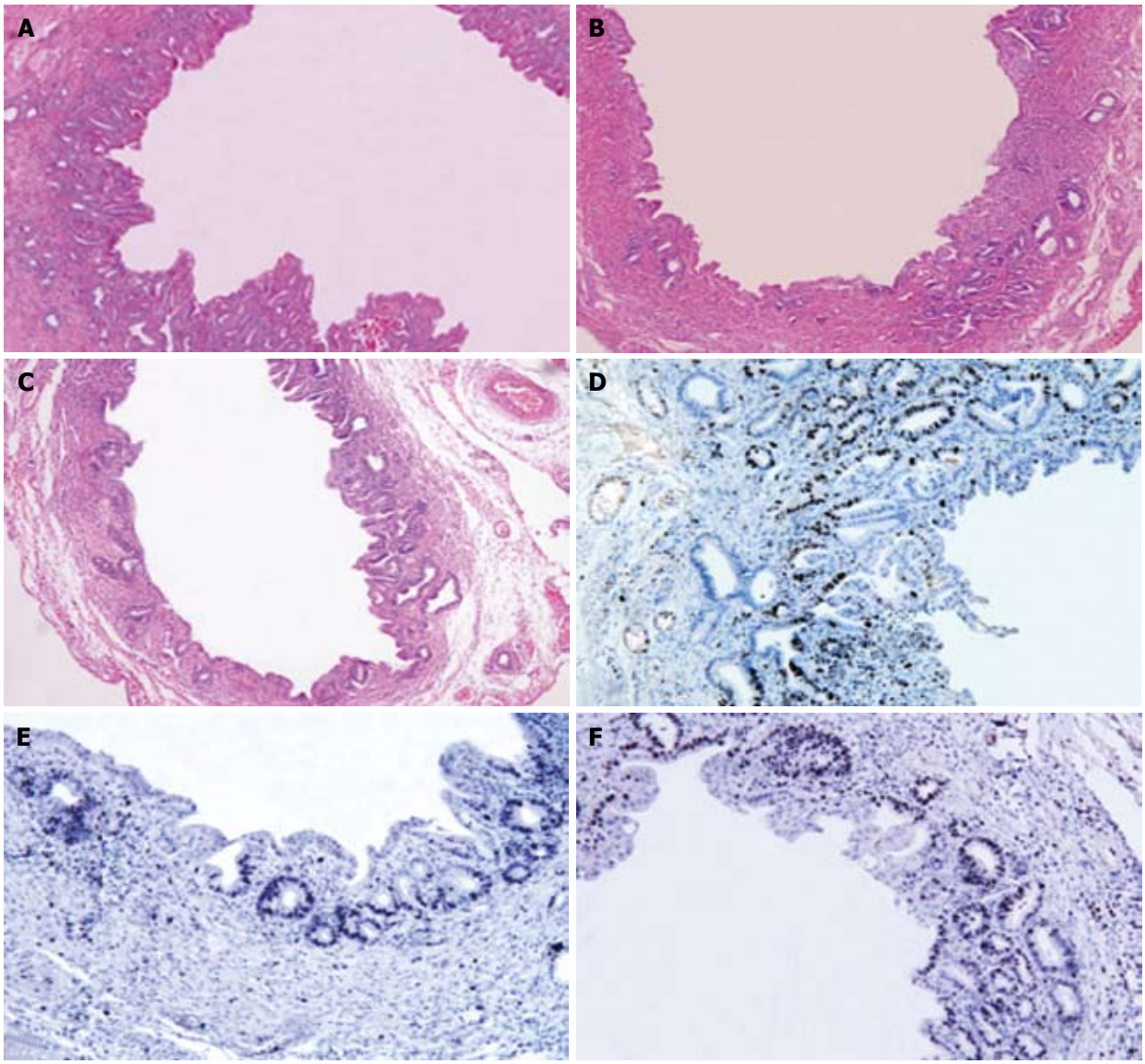

Figure 1 HE staining and immunohistochemistry for c-myc in CPC group (A, D), c-myc shRNA-3 treatment group (B, E), and c-myc shRNA-2 treatment group (C, F). Briefly, treatment with c-myc shRNA, especially with c-myc shRNA-3, can efficaciously inhibit hyperplasia of biliary epithelium, submucosal gland, collagen fiber, and down-regulate c-myc expression (A-C, × 50; D-F, × 100).

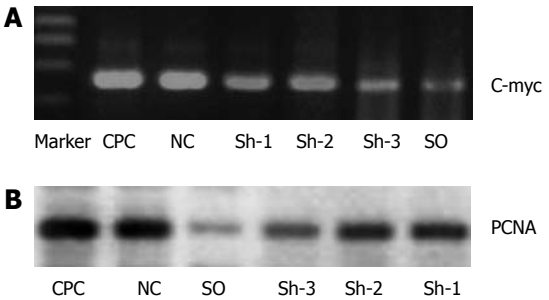

Figure 2 Real-time PCR (A) and Western blot (B) analysis of c-myc and PCNA expression in biliary duct wall.

shRNA, we compared the expression of c-myc, PCNA mRNA and protein in the diseased bile duct wall, revealing a remarkable decrease of c-myc, PCNA mRNA and protein expression in the c-myc shRNA treatment groups ($P < 0.0001$ and $P = 0.001$, respectively), c-myc shRNA-3 treatment group, but still significantly higher than that in the SO group (0.97 ± 0.28 *vs* 0.22 ± 0.09, $P < 0.0001$; 0.82 ± 0.22 *vs* 0.45 ± 0.08, $P = 0.011$). The mRNA and protein levels of c-myc, and PCNA did not differ significantly between the c-myc shRNA-1 and the c-myc shRNA-2 treatment groups. Also, the difference between the CPC group and the negative control group was not statistically significant (Table 1, Figures 1D-F and 2).

Detection of mucin 5AC expression by RT-PCR and immunohistochemistry

To probe the influence of c-myc shRNA on lithogenic potentiality of CPC, RT-PCR and Immunohistochemistry analysis of mucin 5AC were performed, showing a significant decrease of mucin 5AC mRNA and protein expression in the c-myc shRNA treatment groups, which was even more prominent in the c-myc shRNA-3 treatment group (0.42 ± 0.16), when compared with the

Table 1 HYP content and expression level of c-myc, PCNA, mucin5AC, procollagen Ⅲ

	CPC	shRNA-1	shRNA-2	shRNA-3	NC	SO
c-myc/GAPDH	6.21 ± 1.97	2.37 ± 0.77	2.93 ± 0.84	0.97 ± 0.28	6.57 ± 2.11	0.22 ± 0.09
P	< 0.0001	0.001	< 0.0001		< 0.0001	< 0.0001
PCNA/β-actin	3.20 ± 0.81	1.52 ± 0.28	1.83 ± 0.42	0.82 ± 0.22	2.71 ± 0.63	0.45 ± 0.08
P	< 0.0001	0.013	0.005		0	0.011
Mucin5AC/GAPDH	1.87 ± 0.47	0.96 ± 0.28	1.05 ± 0.30	0.42 ± 0.16	1.69 ± 0.41	0.12 ± 0.04
P	< 0.0001	0.004	0.002		< 0.0001	< 0.0001
Procol-Ⅲ/β-actin	4.79 ± 1.27	2.83 ± 0.85	2.39 ± 0.58	1.23 ± 0.35	5.40 ± 1.76	0.52 ± 0.13
P	0	0.001	0.006		< 0.0001	0.001
HYP content	1.29 ± 0.32	0.78 ± 0.16	0.83 ± 0.18	0.55 ± 0.13	1.41 ± 0.36	0.39 ± 0.08
P	0.003	0.041	0.028		0.001	0.038

P value was compared with c-myc shRNA-3 treatment group.

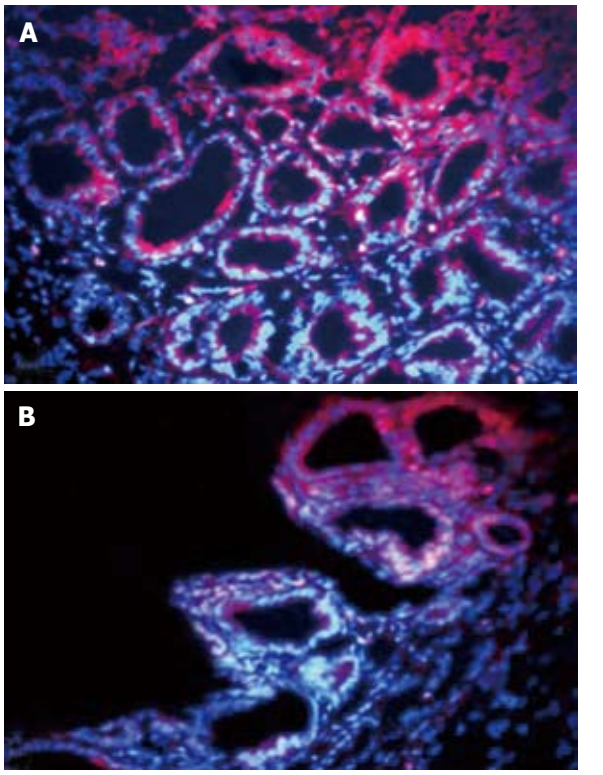

Figure 3 Immunofluorescence (A, B) and RT-PCR (C) analysis of mucin 5AC in bile duct wall. CPC group (A), c-myc shRNA-3 treatment group (B). Briefly, treatment with c-myc shRNA-3 can result in a more prominent down-regulation of mucin 5AC expression (A and B, × 400).

c-myc shRNA-1 and c-myc shRNA-2 treatment groups (0.96 ± 0.28, 1.05 ± 0.30; $P = 0.004$ and $P = 0.002$, Table 1, Figure 3).

Enzymatic histochemical staining of endogenous β-G

To further explore the influence of c-myc shRNA on pigment stone formation, we performed enzymatic histochemical staining of endogenous β-G, which showed a significant increase of endogenous β-G expression in the CPC group. However, β-G expression was significantly decreased in the c-myc shRNA-1, shRNA-2, and shRNA-3 treatment groups, but the difference was not significant in the three groups (Figure 4).

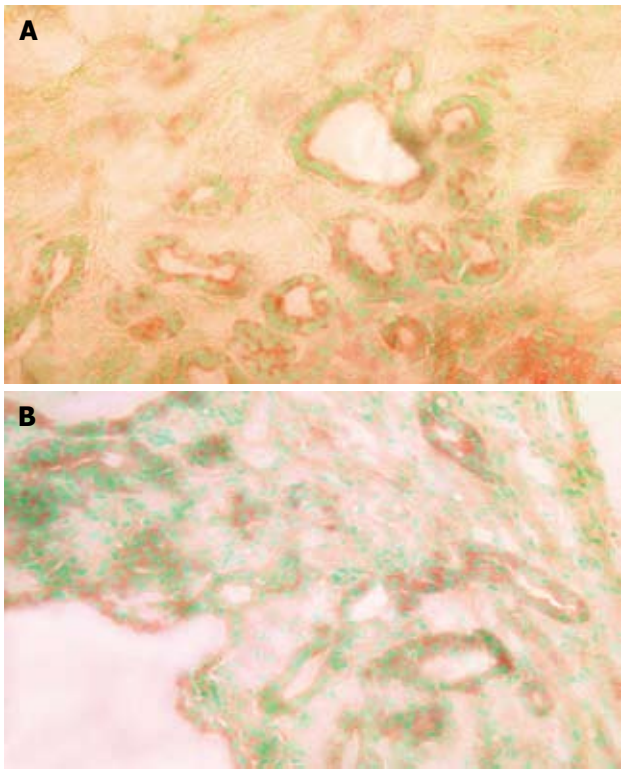

Figure 4 Enzymatic histochemistry staining of endogenous β-G (cryostat section, × 400) in CPC group (A) and c-myc shRNA-3 treatment group (B). A notable reduction of endogenous β-G was observed in the bile duct wall following c-myc shRNA treatment.

Western blot analysis of procollagen Ⅲ in biliary duct wall

To explore the influence of c-myc shRNA on collagen fiber proliferation, we examined the procollagen Ⅲ protein expression in the diseased bile duct. The bile duct in the CPC group displayed a very high level of procollagen Ⅲ protein expression. However, the expression of procollagen Ⅲ protein was significantly decreased after treatment with c-myc shRNA, which was even more prominent in the c-myc shRNA-3 treatment group (1.23

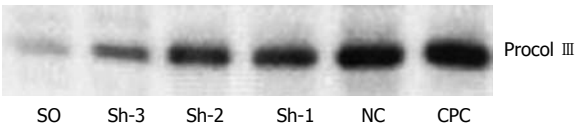

Figure 5 Western blot analysis of procollagen Ⅲ expression in biliary duct wall.

± 0.35 *vs* 2.83 ± 0.85 for the shRNA-1 treatment group, 2.39 ± 0.58 for the shRNA-2 treatment group, $P = 0.001$ and $P = 0.006$), although it was signifcantly higher than that in the SO group (Table 1, Figure 5).

Assessment of hydroxyproline (HYP) content (mg/g of bile duct)

Subsequently, quantitative determination of connective tissue in the diseased bile duct was performed, which showed that the HYP content in the CPC and negative control groups was approximately increased up to three-fold, when compared with the SO group (1.29 ± 0.32 and 1.41 ± 0.36 *vs* 0.39 ± 0.08). However, after treatment with c-myc shRNA, the HYP content was significantly decreased, especially in the c-myc shRNA-3 treatment group (0.55 ± 0.13 *vs* 0.78 ± 0.16 for the shRNA-1 treatment group, 0.83 ± 0.18 for the shRNA-2 treatment group, $P = 0.041$ and 0.028, respectively; Table 1).

DISCUSSION

Since hepatolithiasis is a common disease in Asia and its etiology remains obscure, it is quite difficult to treat and prevent this disease from its lithogenesis. According to its pathology, 75%-100% of hepatolithiasis patients in Asia are characterized by CPC. Increased attention has been paid in recent years to the cause-and-effect relationship of CPC and formation of stones[10,15-17]. Firstly, the stone can not only bring an inflammatory full-thickness penetrating damage to the local biliary duct mucosa in the obstructed part, but also lead to a down-stream mucosal injury in the distant bile duct as the stone and inflamed bile move downwards. Therefore, damage to the related biliary ducts caused by the stone is extensive, and not merely localized to the stone resident part. This is why stone-induced CPC exists widely after removal of the stone[11-16]. On the other hand, recurrent attacks of CPC would in turn facilitate formation of new stones by causing such lithogenic pathology changes as biliary stricture and biliary infection. Furthermore, recurrent CPC can be directly involved in formation of stones by producing mucoglycoproteins secreted by the proliferated submucosal gland. As we know, mucoglycoprotein is not only a contributing factor for intrahepatic stones but also a protein that can directly participate in formation of stone nucleation or reticular lithogenesis framework[23-26]. As mentioned above, a vicious cycle of CPC, biliary stricture and stone may develop. It is thereby reasonable that treatment of heaptolithiasis should be directed not only at removal of the stone and correction of the biliary stricture, but also at control of postoperative CPC, a key factor for this vicious cycle[12-16,19,20].

Recently, proto-oncogene c-myc has become an attractive target for anti-proliferative treatment of hypernomic proliferation diseases, because it is at the center of a transcription factor network that regulates cellular proliferation, replication, growth, differentiation, and apoptosis[27-30]. Specific blockage of the c-myc gene expression can partially inhibit cellular proliferation, thus efficiently preventing vascular restenosis after angioplasty by inhibiting endangium cell proliferation[27,28]. In this study, we also investigated the anti-proliferative effectiveness of c-myc shRNA on hypernomic proliferation behavior of CPC[17,18,29]. As expected, HE staining, immunohistochemistry, RT-PCR and Western blotting showed that treatment with c-myc shRNA could efficiently inhibit hyperplasia of the biliary epithelium, submucosal gland and collagen fiber by specifically blocking mRNA and protein expression of the proliferation-related gene c-myc and PCNA. The levels of c-myc, and PCNA mRNA and protein expression were much lower in the c-myc shRNA-3 treatment group than in the c-myc shRNA-1 and shRNA-2 treatment groups, which indicates that c-myc shRNA-3 may have a better anti-proliferative effect on CPC[9,15,27-30].

To analyze the effect of this gene therapy on the lithogenic potentiality of CPC, we compared the expression of mucin5AC and endogenous β-G in the diseased bile duct. Among the nine mucoglycoproteins identified so far, up-regulation of mucin5AC expression is considered to be closely related to the formation of stones[25,31], which is consistent with our findings. In the present study, the expression of mucin5AC mRNA and protein was significantly increased in the CPC group. However, the expression of mucin5AC was obviously decreased after treatment with c-myc shRNA, especially after treatment with c-myc shRNA-3, which suggests that c-myc shRNA can effectively inhibit the inactivation of such mucin genes as mucin5AC and secretion of muglycoprotein. It is noteworthy that reduced muglycoprotein helps decrease bile viscidity and aggregation or sedimentation of lithogenic ingredients in the bile, which might be significant in preventing stone recurrence[24,26,32]. The expression of endogenous β-G in the diseased bile duct was also obviously decreased after treatment with c-myc shRNA, which may be explained by the inhibitory effect of c-myc shRNA on the proliferation of biliary epithelium and submucosal gland. The inhibitory effect of c-myc shRNA on endogenous β-G would, to some degree, be helpful in preventing postoperative biliary stone recurrence[21,33].

Considering the potential anti-proliferative effect of c-myc shRNA on collagen fiber proliferation, c-myc shRNA treatment may prevent biliary tract restenosis secondary to CPC[8,9]. In our study, HE staining showed that collagen fiber proliferation was significantly lower in the diseased bile duct after treatment with c-myc shRNA than CPC, which suggests that the incidence of biliary tract stricture secondary to CPC can be reduced. Further comparison displayed that treatment with c-myc shRNA-3 demonstrated a better inhibitory effect on procollagen Ⅲ protein and HYP content than treatment

with shRNA-2 and shRNA-1, which indicates that c-myc shRNA-3 has a bright future in preventing bile duct fibrosis and biliary stricture[20,28,29,34].

In conclusion, anti-proliferative treatment with c-myc shRNA is likely to open a new feasible approach to the treatment of postoperative residual CPC. Furthermore, the inhibitory effects of c-myc shRNA on the lithogenic potentiality of CPC can assist in reducing postoperative recurrence of intrahepatic calculi. More importantly, this novel treatment would lay an experimental foundation of development of drugs for preventing stone recurrence after choledochoscopic lithotomy, at least in part, and reducing the incidence of reoperation and choledochoscopic lithotomy[2,13-18,20,30]. However, further study is needed on its long-term effect, related complications, and more efficient gene expression vectors before its clinical application[13,33,35].

COMMENTS

Background

In recent years, with a deeper understanding of the pathological changes in hepatolithiasis, the high stone recurrence rate has gradually been recognized, and is currently considered due to the postoperative chronic proliferative cholangitis (CPC). In this study, we investigated the inhibitory effect of c-myc shRNA on hyperplastic behavior and lithogenic potentiality of CPC.

Research frontiers

Multiple factors for lithogenesis of intrahepatic stones have brought enormous difficulties to its prevention and treatment and 75%-100% of hepatolithiasis patients in the Asian-Pacific regions are pathologically characterized by CPC, a key factor for preventing calculus recurrence. Treatment of CPC after operation might assist in increasing the curative effect on hepatolithiasis.

Innovations and breakthroughs

The high recurrence rate of intrahepatic stones is still a problem to be solved in hepatobiliary surgery. Since there is no effective medication for preventing stone recurrence after choledochoscopic lithotomy and for testing its pathology, stone recurrence and reoperation cannot be avoided. Our preliminary results showed that c-myc shRNA can inhibit hyperplastic behavior and lithogenic potentiality of CPC, thus laying an experimental foundation of development of drugs for preventing stone recurrence.

Applications

Intralumenal administration of c-myc shRNA is a promising therapeutic approach to CPC, and might assist in reducing the lithogenic potentiality of CPC.

Peer review

The authors of this paper investigated the efficacy of c-myc shRNA in ameliorating histological and molecular manifestations in an animal model of hepatolithiasis. The study was well designed and its findings are interesting and informative.

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