Journal of Toxicology and Environmental Health Sciences Vol. 2(2) pp. 17-23, July 2010
Available online at http://www.academicjournals.org/JTEHS
ISSN 2006-9820 ©2010 Academic Journals

*Full Length Research Paper*

# The correlation between the malignant proliferation on cholangiocarcinoma cell lines and in bile from *Helicobacter pylori* infected patients with biliary tract stones

**Xudong Xu[1], Quan Sun[2], Zhisu Liu[2], Lin Zhang[1], Zhiyong Luo[1], Yun Xia[1] and Yaqun Wu[1]***

[1]Department of General Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, P.R. China
[2]Department of General Surgery, Zhongnan Hospital, Wuhan University, Wuhan 430071, P.R. China.

Accepted 25 May, 2010

***Helicobacter pylori* has been detected in human tissue and is a candidate for etiologic investigations on the causes of hepatic and biliary tract diseases, but reliable serologic tests need to be developed in order to pursue such investigations. The aim of this study was to assess the correlation between the infection of *H. pylori* in bile from patients with biliary tract stones and the proliferation of human cholangiocarcinoma and its mechanism. Choledocholithiasis bile with *positive H. pylori (PCB),* Choledocholithiasis bile with *negative H. pylori* (NCB) and *normal bile* (NB) were part of the study. Cholangiocarcinoma cell lines QBC939 and TFK-1 were analyzed. The proliferative effects were measured by methabenzthiazuron (MTT) assay. Cell cycle and apoptosis were analyzed by flow cytometry. Compared with NB and NCB, PCB significantly promoted the proliferation of cholangiocarcinoma cell lines QBC939 and TFK-1. The proliferative index in PCB group was obviously higher than that in NCB group after being treated with 1% PCB for 48 h ($p < 0.05$). As far as the apoptosis rate was concerned, there were no obvious differences between PCB group and NCB group ($p < 0.05$), same as between PCB group and NB group ($p < 0.05$). The percentage of S phase increased remarkably in PCB group compared with NCB group, while the percentage of $G_0/G_1$ phase decreased remarkably in PCB group compared with NCB group. It was suggested that PCB can greatly promote the malignant proliferation of human cholangiocarcinoma cell lines QBC939 and TFK-1, and the mechanism was affected by the changes of cell cycle. So we can predict that there was perhaps a close relation between *H. pylori* infection and cholangiocarcinoma.**

**Key words:** Malignant proliferation, *Helicobacter pylori*, cholelithiasis, bile, cholangiocarcinoma.

## INTRODUCTION

Cholangiocarcinoma (CCA) is a devastating malignancy that appears late, which is notoriously difficult to diagnose, and is associated with a high mortality rate (Briggs et al., 2009). The incidence of intrahepatic cholangiocarcinoma is increasing worldwide. The cause of this rise is unclear, although it could be related to an interplay between pre-disposing genetic factors and environmental triggers. CCA was first reported by Durand-Fardel in 1840 (Olnes and Erlich, 2004). The tumor arises from the ductular epithelium of the biliary tree, either within the liver (intrahepatic CCA) or more commonly from the extrahepatic bile ducts (extrahepatic CCA). The disease is usually fatal because of its late clinical presentation and the lack of effective non-surgical therapeutic modalities (Hong et al., 2009). Most patients have unresectable disease at presentation

*Corresponding author, E-mail: wyqmd@126.com. Tel: 86-27-83663819. Fax: +86-27-83646605.

and die within 12 months from the effects of cancer cachexia and a subsequent rapid decline in performance status. Overall survival rate including resected patients is poor, with less than 5% of patients surviving to 5 years, which has not changed significantly over the past 30 years (Shaib and El-Serag, 2004). Although CCA is a relatively rare tumor, interest in this disease is rising as incidence and mortality rates increase markedly (Khan et al., 2002). In recent years, the etiology research on CCA has been the focus of attention (Anderson et al., 2004; Chen et al., 2005; Enjoji et al., 2005). According to the epidemiology data, cholelithiasis is a dangerous factor to CCA, and the infection of *Helicobacter pylori* is closely related to the occurrence of cholelithes and chronic inflammation of biliary epithelium (Boomkens et al., 2004; Chang et al., 2005; Monstein et al., 2002; Tsai et al., 2004).

*H. pylori* is a noninvasive, nonspore-forming, and spiral-shaped Gram-negative rod bacteria measuring approximately 3.5 × 0.5 μm. This bacterium induces infiltration of the biliary mucosa by neutrophils, macrophages, and T and B lymphocytes. However, this immune and inflammatory response to *H. pylori* infection suggests there is an increase in both epithelial all proliferation, as well as cell death by apoptosis. It is transmitted from human to human, usually in early childhood, and it is always transferred to biliary tract by oddi sphincter. It colonizes the biliary epithelium for a lifetime in the absence of specific antimicrobial therapy. Recent studies reveal that *H. pylori* injects bacterial proteins which greatly damage biliary epithelium and cause chronic inflammation to the cytosol of the host cell and regulates the intracellular signal transduction in the host cell (Boomkens et al., 2005). This mechanism provides a noble means of resolving how *H. pylori* survives in human biliary tract. Some molecular studies have recently shown that *H. pylori* DNA and intracellular expression of *H. pylori* virulence genes are still detectable in biopsies of patients with precancerous lesions (Clyne and Drumm, 1996). *H. pylori* infection's ability to foster a chronic inflammatory response is the best explanation of the bacterium's carcinogenic potential. Preliminary experiments testified that the proliferation of CC cell lines QBC939 and TFK-1 had something to do with the infection of *H. pylori*, but other CC cell lines did not have type characteristics of CCA like QBC939 and TFK-1. Therefore we conducted the study to emphasize the discussion on the proliferative effects of choledocholithiasis bile (CB) infected by *H. pylori* on the culture *in vitro* of human cholangiocarcinoma cell lines QBC939 and TFK-1.

## MATERIALS AND METHODS

### Collection and treatment of samples

The experimental objects are divided in 3 groups, *H. pylori* positive choledocholithiasis bile (PCB) group, *H. pylori* negative choledocholithiasis bile (NCB) group, and normal bile (NB) group, each taking 120 samples. The 120 *H. pylori* PCB samples are selected from surgical patients in Tongji Hospital, including 67 males, 53 females, aged between 24 and 62. Among the sample patients of this group, 78 samples have choledocholith with calculus of intrahepatic duct and 42 samples have simple choledocholith. The 120 *H. pylori* NCB samples are from surgical patients in Tongji Hospital, including 81 males, 39 females, aged between 25 and 58. Among the sample patients of this group, 42 samples have choledocholith with calculus of intrahepatic duct, 57 samples have simple choledocholith, and 21 samples have choledocholith with cholelithiasis. The 120 NB samples are from patients without liver or gallbladder diseases during the corresponding period, including 71 males and 49 females, aged between 25 and 56. All patients are informed of relative information and they all express consent and show their cooperation in our study. Bile is sampled during the examination or during the operation because of hepatobiliary diseases. Especially in reference to normal bile, samples are taken from normal people during the examination or in other diseases during the operation. The choledocholithiasis bile (CB) samples have all passed ELISA test and PCR test. The type of ELISA is double antibody sandwich. The PCR primers used in this study are 5'-GCCAATGGTAAATTAGTT-3'(ureA1) and 5'-CTCCTTAATTGTTTTTAC-3' (ureA2). UreA1 and ureA2 primers are amplified 411 bp fragment of the ureA gene. The cycles consist of 2 min at 94℃ for the first cycle, 1 min at 94℃, 1 min at 42℃ and 1 min at 72℃ for the next 40 cycles and 10 min at 72℃ for the last cycle. The PCR products are analyzed by means of electrophoresis of a 20 μl aliquot through a 1% agarose gel containing 0.5 μg/ml ethidium bromide. If results from both tests are positive, the sample will be defined as *H. pylori* positive; otherwise, it will be defined as *H. pylori* negative(Gieseler et al., 2005; Rahn et al., 2004). And results from both tests are negative in normal bile. The bile samples are centrifuged rapidly at a speed of 4000 rpm for 5 min after extraction. The supernatant is taken and reserved in the refrigerator at –80 (Iwata et al., 2002; Zampa et al., 2004).

### Cell culture and reagent

These studies are performed with human CCA cell lines QBC939 and TFK-1. Human CCA cell lines QBC939 and TFK-1 were established and donated by Professor Wang Shuguang from Southwest Hospital of Third Military Medical University. They were constructed with cloning technique such as recombinant plasmid and so on. The CCA cell lines QBC939 and TFK-1 have type characteristics of CCA which is highly invasive. RPM11640 culture medium and 10% fetal calf serum are purchased from the American Gibco Industries, Inc. Meanwhile, penicillin and streptomycin are added into culture medium. The volume ratios are both 1 versus 1000.

### Cytotoxicity test

We test the cytotoxicity of the gradient dilution from the bile samples (Kanbar et al., 2004; Ricci et al., 2002; Yao et al., 2005). When the final concentration of the samples is 1% (10 μl bile sample/ml culture medium), there will be no obvious toxicity to CCA cell lines QBC939 and TFK-1. And 1% samples are used for cytotoxicity test.

### Methabenzthiazuron (MTT) assay

We used 2.5 g/L trypsin to digest CCA cell lines QBC939 and TFK-1 in logarithmic growth phase, making single-cell suspension. CCA cell lines QBC939 and TFK-1 are inoculated in 96-well plates and cell counting gives a number of $1.0\times10^5$ cells/ml. 1%PCB, 1%NCB,

**Table 1.** Variation of optical density (OD 490 nm) values at different time points during the co-culture of choledocholithiasis bile and QBC939.

| Time | OD 490 nm | | |
|---|---|---|---|
| | NB | NCB | PCB |
| 1 d | 0.15±0.02 | 0.19±0.03 | 0.20±0.03 |
| 2 d | 0.20±0.03 | 0.33±0.04 * # | 0.48±0.05 **#Δ |
| 3 d | 0.24±0.03 | 0.52±0.06 * # # | 0.82±0.09 **# #ΔΔ |

Note: compared with 1 d, $^{*}p < 0.05$, $^{**}p < 0.01$; compared with NB group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; compared with NCB group, $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$.

**Table 2.** Variation of optical density (OD 490 nm) values at different time points during the co-culture of choledocholithiasis bile and TFK-1.

| Time | OD 490 nm | | |
|---|---|---|---|
| | NB | NCB | PCB |
| 1 d | 0.14±0.02 | 0.18±0.03 | 0.21±0.03 |
| 2 d | 0.22±0.04 | 0.35±0.06 * # | 0.47±0.08 **#Δ |
| 3 d | 0.25±0.04 | 0.51±0.06 * # # | 0.83±0.11 **# #ΔΔ |

Note: compared with 1 d, * $p<0.05$, ** $p < 0.01$; compared with NB group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; compared with NCB group, $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$.

1%NB are added respectively after cells are adhered. After incubiting for 1 d (24 h), 2 d (48 h), 3 d (72 h) respectively, 20 µl of MTT stock solution (4 mg/ml) is added to each well, containing about 0.25 ml medium, and the mixture is incubated for 3 h. The supernatant solution is removed with a pipette. 150 µl of DMSO is added rapidly to each well (within 3 s) at 37 and oscillated for 5 min in order to fully dissolve the crystallisate. The MTT solution is carefully decanted off and formazan is extracted from the cells in each well. The optical density, OD value (490 nm) is detected on the enzymes immunoassay analyzer (Pouns et al., 2009). All MTT assays are tripled.

### Cell cycle and apoptosis analyzed by flow cytometry

The flow cytometer used is a Coulter EPICS-XL (Coulter, Miami, FL, USA). Instrument calibration is performed daily according to the recommendation of the manufacturer. An average of $1\times10^4$ cells from each sample are counted in the flow cytometer. After incubated with 1%PCB, 1%NCB, and 1%NB for 48 h, QBC939 and TFK-1 cell solutions are respectively collected to centrifuge at 800 rpm for 5 min. Then, they are fixed by 80% ethanol pre-cooled at -20℃. The cells are incubated in the dark at room temperature and then diluted to 1 ml in harvest buffer and stored in ice until FACS analysis. To differentiate viable from dead cells, 1 mg/ml propidium iodide is added to samples approximately 3 min before analysis. This protocol works for freshly prepared human cells and cell lines. Cell cycle and apoptosis analysis are measured by flow cytometry (Tian et al., 2009). The same condition is used in order to ensure the credibility of experimental results. When cell cycle is arrested in $G_0/G_1$ phase, cell apoptosis is strengthened and we can see blue particulate fluorescence signal with the help of fluorescence microscopy.

### Statistical analysis

Mean±SD was used for measurement of data; all the data are analyzed with t-test in Statistical Package for Social Science (SPSS11.5, license code: 30001359390), and independent t-test is selected to analyze the data. The confidence interval is calculated at the 95% level. All P values are 2-tailed and P values <0.05 are considered to be statistically significant.

## RESULTS

### Effects of choledocholithiasis bile (CB) infected by helicobacter pylori on the proliferation of QBC939 and TFK-1 Cells

After co-culturing bile and CCA cell lines for 1 d, the variation of optical density among PCB group, NCB group and NB group shows no significant difference ($p > 0.05$), the difference between PCB group and NCB group is not significant either ($p > 0.05$). After co-culturing bile and CCA cell lines for 2 d, the variation of optical density among PCB group, NCB group and NB group shows significant difference ($p < 0.05$), and the difference between PCB group and NCB group also shows significance ($p < 0.05$). After co-culturing bile and CCA cell lines for 3 d, the variation of optical density among PCB group, NCB group and NB group shows significant difference ($p < 0.01$), and the difference between PCB group and NCB group also shows significance ($p < 0.01$). Therefore both PCB group and NCB group can promote the proliferation of QBC939 and TFK-1 cells. This promotion is time dependent. Compared with NCB group, PCB group has a more apparent promotional function on the proliferation of human CCA cell lines QBC939 and TFK-1 ($p < 0.05$) (Tables 1 and 2).

### Cell cycle and apoptosis analyzed by flow cytometry

Proliferative indexes (PI) of PCB group, NCB group and NB group about CCA cell line QBC939 are 72±6, 50±4 and 30±3, respectively (Figure 1). And the proliferative indexes (PI) of PCB group, NCB group and NB group about CCA cell line TFK-1 are 74±7, 49±4 and 28±3 respectively (Figure 2). Compared with NB group, the difference within PCB group and NCB group has prominent significance ($p < 0.05$), and the difference between the two groups also has marked significance ($p < 0.05$), $PI = (S+G_2/M)$. The proportion of CCA cell line QBC939 in PCB group and NCB group in S phase are 58±5% and 38±4% respectively, which shows a marked increase on the basis of NB group 20±3%, and the difference between PCB group and NCB group shows significance ($p < 0.01$) (Figure 3). The proportion of CCA

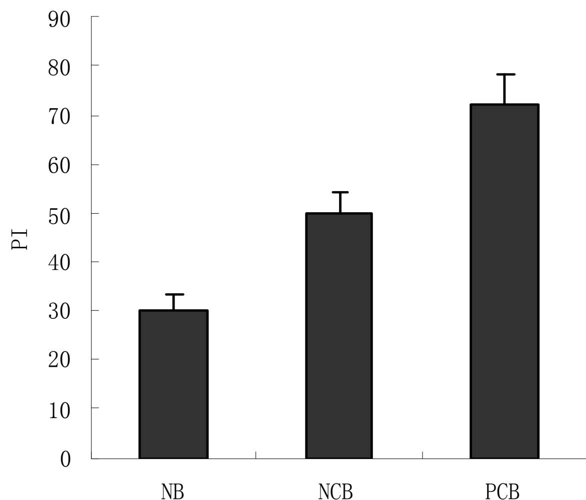


**Figure 1.** Comparison of proliferative indexes (PI) about QBC939 cell.

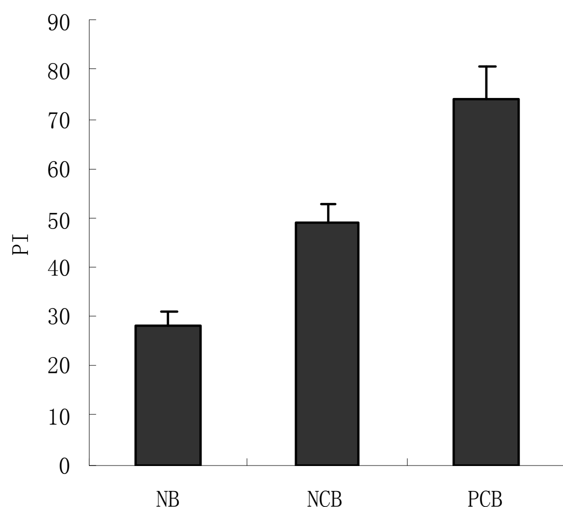


**Figure 2.** Comparison of proliferative indexes(PI) about TFK-1 cell.

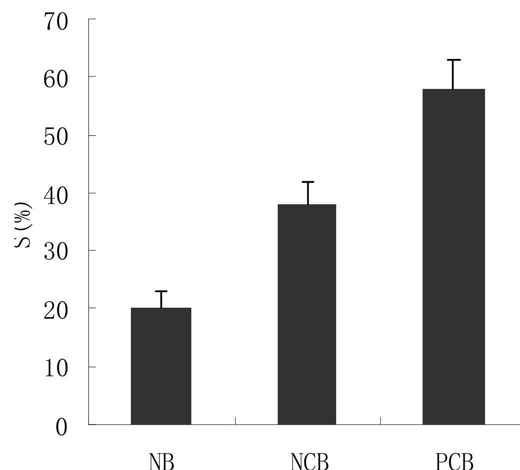


**Figure 3.** Comparison of cell proportions in S phase about QBC939 cell.

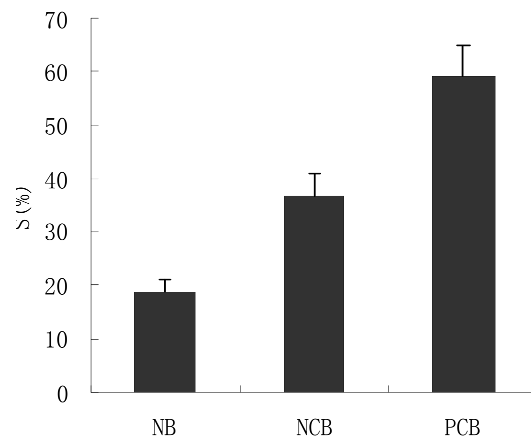


**Figure 4.** Comparison of cell proportions in S phase about TFK-1 cell.

cell line TFK-1 in PCB group and NCB group in S phase are 59±6% and 37±4% respectively, which shows a marked increase on the basis of NB group 19±2%, and the difference between PCB group and NCB group shows significance ($p < 0.01$) (Figure 4). The proportion of CCA cell line QBC939 in PCB group and NCB group in $G_0/G_1$ phase are 28±3% and 48±5% respectively, which shows a remarkable decrease on the basis of NB group 68±8%, and the difference between PCB group and NCB group shows significance ($p < 0.01$) (Figure 5). The proportion of CCA cell line TFK-1 in PCB group and NCB group in $G_0/G_1$ phase are 29±3% and 51±6% respectively, which shows a remarkable decrease on the basis of NB group 70±8%, and the difference between PCB group and NCB group shows significance ($p < 0.01$) (Figure 6). The apoptosis rates (AR) of QBC939 cell in PCB group, NCB group and NB group are 22±3%, 28±6% and 24±4% respectively (Figure 7), while the apoptosis rates (AR) of TFK-1 cell in PCB group, NCB group and NB group are 23±3%, 27±6% and 25±4% respectively (Figure 8), the comparison among the three groups shows no significant difference ($p > 0.05$).

## DISCUSSION

The vast majority of experts and scholars acknowledge that *H. pylori* could cause cell cancerization through inducing the activation of oncogene and inactivation of anti-oncogene (Nakajima et al., 2008; Nakajima et al., 2009). Since Lin et al. (1995) employed PCR method in finding *H. pylori* in the bile duct of patients with carcinoma of the head of the pancreas and CCA for the first time in

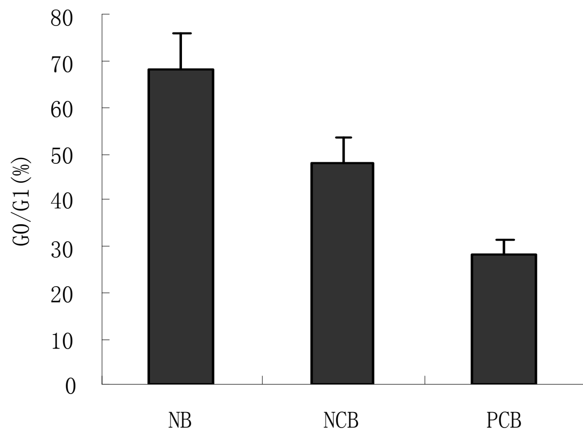


**Figure 5.** Comparison of cell proportions in $G_0/G_1$ phase about QBC939

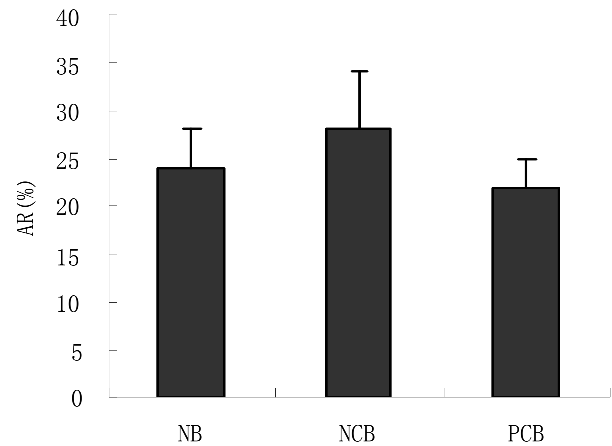


**Figure 7.** Comparison of apoptosis rates about QBC939.

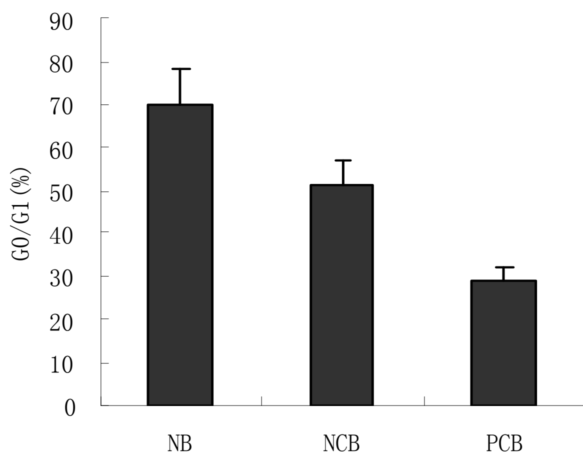


**Figure 6.** Comparison of cell proportions cell in $G_0/G_1$ phase about TFK-1 cell.

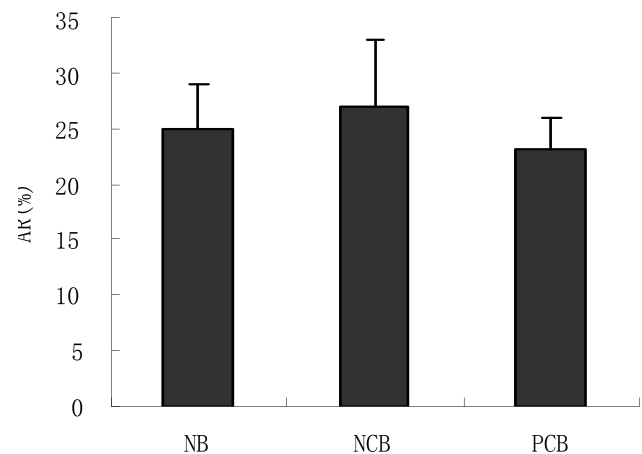


**Figure 8.** Comparison of apoptosis rates cell about TFK-1 cell.

1995; the relation between *H. pylori* and malignant disease of biliary tract has drawn people's attention. In 2000, Paziak-Domanska et al. (2000) co-cultured cagA$^+$ *H. pylori* mutant strain at certain concentration and lymphocyte in peripheral blood of healthy people for 96 h, and employed flow cytometry in measuring T lymphocyte and B lymphocyte in the culture media. They found that cagA$^+$ *H. pylori* strain could significantly inhibit the proliferation of T lymphocyte, namely, cagA$^+$ *H. pylori* has the ability to inhibit the immune function of human cells, which led to the further deduction that *H. pylori* could indirectly promote the occurrence and development of CCA through hypoimmunity and reducing the immunity monitoring function. In the meantime, a lot of scholars have explored the relation of other bacteria or cytokine present in bile and CCA. Until now, there is not an identical conclusion. No evidence is shown that CCA has something to do with other bacteria or cytokine. In 2002, Matsukura et al. (2002) measured the bile of patients with benignant and malignant biliary tract diseases, and found *H. bilis* closely related to CCA and other malignant biliary tract diseases in some populations, while it has nothing to do with benign biliary tract diseases. Further studies suggest that there is a relationship between bacteria load and the effect on CCA cells proliferation and apoptosis. It is also confirmed by preliminary experiment. In a way, the changes of QBC939 and TFK-1 cells proliferation and apoptosis are on the premise of bacteria load. That is to say, there are perhaps some intimidating correlations between *H. pylori* infection of biliary tract and CCA. However, there are no clear conclusions for the mechanisms of malignancy of biliary tract cells induced by *H. pylori* infection. It needs further intensive research.

The results from this experiment indicate that both PCB group and NCB group could promote the proliferation of CCA cell lines, but the proliferative effects of PCB group are more obvious than that of NCB group. That is to say, patients with *H. pylori* positive cholelithiasis are more

liable to develop CCA. This experimental result can well explain the phenomena such as the high prevalence rate of CCA among patients with cholelithiasis and the uneven probability of CCA among patients with cholelithiasis. Therefore, *H. pylori* infection of biliary tract perhaps plays a role in the occurrence and development of CCA. It is yet to be studied and there is not a consistent conclusion about the causes of CCA (Boberg et al., 2006).

How does *H. pylori* infection promote the proliferation of human CCA QBC939 and TFK-1 cells? Flow cytometry is adopted in analyzing cell cycle and apoptosis in this study. The analysis of cell cycle indicates that, compared with NB group, the proliferative indexes of QBC939 and TFK-1cells increased obviously after being treated with PCB group and NCB group for 48 h, the percentage of S phase increased markedly, while the percentage of $G_0/G_1$ phase decreased remarkably, and the difference between PCB group and NCB group is significant. Data of cell apoptosis shows that PCB group and NCB group have no obvious impact on the apoptosis rate of QBC939 and TFK-1 cells. This group study verifies that bile from patients with choledocholithiasis infected by *H. pylori* promotes the proliferation of CCA cell lines QBC939 and TFK-1 through changing the cell cycle rather than the cell apoptosis.

This study conducts a tentative investigation of the relation between choledocholithiasis and CCA and the internal relation between *H. pylori* infection and occurrence and development of CCA, which illustrates the mechanism that choledocholithiasis bile infected by *H. pylori* could promote the proliferation of CCA cell through changing the cell cycle. Further research is still needed in regards to how *H. pylori* infection induces the changing of the cell cycle.

## ACKNOWLEDGEMENTS

The work was supported by the National Natural Science Foundation of China (No. 30672426 and No. 30570908), and Project of Scientific Commission of Hubei province (No. 2003AA301C27 and No. 2004AA301C28).

### REFERENCES

Anderson CD, Rice MH, Pinson CW, Chapman WC, Chari RS, Delbeke D (2004). Fluorodeoxyglucose PET imaging in the evaluation of gallbladder carcinoma and cholangiocarcinoma. J. Gastrointest. Surg. 8: 90-97.

Boberg KM, Jebsen P, Clausen OP, Foss A, Aabakken L, Schrumpf E (2006). Diagnostic benefit of biliary brush cytology in cholangiocarcinoma in primary sclerosing cholangitis. J. Hepatol. 45: 568-574.

Boomkens SY, de Rave S, Pot RG, Egberink HF, Penning LC, Rothuizen J, Zondervan PE, Kusters JG (2005). The role of Helicobacter spp. in the pathogenesis of primary biliary cirrhosis and primary sclerosing cholangitis. FEMS Immunol. Med. Microbiol. 44: 221-225.

Boomkens SY, Kusters JG, Hoffmann G, Pot RG, Spee B, Penning LC, Egberink HF, van den Ingh TS, Rothuizen J (2004). Detection of Helicobacter pylori in bile of cats. FEMS Immunol. Med. Microbiol. 42:307-311.

Briggs CD, Neal CP, Mann CD, Steward WP, Manson MM, Berry DP (2009). Prognostic molecular markers in cholangiocarcinoma: a systematic review. Eur. J. Cancer. 45: 33-47.

Chang FY, Chen CY, Lu CL, Luo JC, Lu RH, Lee SD (2005). Response of blood endothelin-1 and nitric oxide activity in duodenal ulcer patients undergoing Helicobacter pylori eradication. World J. Gastroenterol. 11: 1048-1051.

Chen CY, Lin XZ, Wu HC, Shiesh SC (2005). The value of biliary amylase and Hepatocarcinoma-Intestine-Pancreas/Pancreatitis-associated Protein I (HIP/PAP-I) in diagnosing biliary malignancies. Clin. Biochem. 38: 520-525.

Clyne M, Drumm B (1996). Cell envelope characteristics of Helicobacter pylori: their role in adherence to mucosal surfaces and virulence. FEMS Immunol. Med. Microbiol. 16: 141-155.

Enjoji M, Nakamuta M, Yamaguchi K, Ohta S, Kotoh K, Fukushima M, Kuniyoshi M, Yamada T, Tanaka M, Nawata H (2005). Clinical significance of serum levels of vascular endothelial growth factor and its receptor in biliary disease and carcinoma. World J. Gastroenterol. 11: 1167-1171.

Gieseler S, Konig B, Konig W, Backert S (2005). Strain-specific expression profiles of virulence genes in Helicobacter pylori during infection of gastric epithelial cells and granulocytes. Microbes. Infect. 7: 437-447.

Hong SM, Pawlik TM, Cho H, Aggarwal B, Goggins M, Hruban RH, et al. (2009). Depth of tumor invasion better predicts prognosis than the current American Joint Committee on Cancer T classification for distal bile duct carcinoma. Surgery, 146: 250-257.

Iwata K, Shijo H, Kamimura S, Uehara Y, Kitamura Y, Iida T, Okada Y, Akiyoshi N, Watanabe H, Sakisaka S (2002). Effects of esophageal varices obliteration by endoscopic variceal sclerotherapy on asialoscintigraphy and liver function test. Hepatol. Res. 22: 45-51.

Kanbar G, Engels W, Nicholson GJ, Hertle R, Winkelmann G (2004). Tyramine functions as a toxin in honey bee larvae during Varroa-transmitted infection by Melissococcus pluton. FEMS Microbiol. Lett. 234: 149-154.

Khan SA, Taylor-Robinson SD, Toledano MB, Beck A, Elliott P, Thomas HC (2002). Changing international trends in mortality rates for liver, biliary and pancreatic tumours. J. Hepatol. 37: 806-813.

Lin TT, Yeh CT, Wu CS, Liaw YF (1995). Detection and partial sequence analysis of Helicobacter pylori DNA in the bile samples. Dig. Dis. Sci. 40: 2214-2219.

Matsukura N, Yokomuro S, Yamada S, Tajiri T, Sundo T, Hadama T, Kamiya S, Naito Z, Fox JG (2002). Association between Helicobacter bilis in bile and biliary tract malignancies: *H. bilis* in bile from Japanese and Thai patients with benign and malignant diseases in the biliary tract. Jpn. J. Cancer Res. 93: 842-847.

Monstein HJ, Jonsson Y, Zdolsek J, Svanvik J (2002). Identification of Helicobacter pylori DNA in human cholesterol gallstones. Scand. J. Gastroenterol. 37: 112-119.

Nakajima T, Enomoto S, Ushijima T (2008). DNA methylation: a marker for carcinogen exposure and cancer risk. Environ. Health Prev. Med. 13: 8-15.

Nakajima T, Yamashita S, Maekita T, Niwa T, Nakazawa K, Ushijima T (2009). The presence of a methylation fingerprint of Helicobacter pylori infection in human gastric mucosae. Int. J. Cancer. 124: 905-910.

Olnes MJ, Erlich R (2004). A review and update on cholangiocarcinoma. Oncology, 66: 167-179.

Paziak-Domanska B, Chmiela M, Jarosinska A, Rudnicka W (2000). Potential role of CagA in the inhibition of T cell reactivity in Helicobacter pylori infections. Cell Immunol. 202:136-139.

Pouns O, Mangas A, Covenas R, Geffard M (2009). Circulating antibodies directed against "polycyclic aromatic hydrocarbon-like" structures in the sera of cancer patients. Cancer Epidemiol. 33: 3-8.

Rahn W, Redline RW, Blanchard TG (2004). Molecular analysis of Helicobacter pylori-associated gastric inflammation in naive versus previously immunized mice. Vaccine 23: 807-818.

Ricci V, Sommi P, Fiocca R, Necchi V, Romano M, Solcia E (2002). Extracellular pH modulates Helicobacter pylori-induced vacuolation and VacA toxin internalization in human gastric epithelial cells.

Biochem. Biophys. Res. Commun. 292: 167-174.
Shaib Y, El-Serag HB (2004). The epidemiology of cholangiocarcinoma. Semin. Liver. Dis. 24: 115-125.
Tian HL, Zhao D, Ren LM, Su XH, Kang YH (2009). Effects of (-)doxazosin on histomorphologic and cell apoptotic changes of the hyperplastic prostate in castrated rats. Am. J. Med Sci. 338: 196-200.
Tsai CC, Huang LF, Lin CC, Tsen HY (2004). Antagonistic activity against Helicobacter pylori infection in vitro by a strain of Enterococcus faecium TM39. Int. J. Food Microbiol. 96: 11-12.
Yao Q, Chen J, Cao H, Orth JD, McCaffery JM, Stan RV, McNiven MA (2005). Caveolin-1 interacts directly with dynamin-2. J. Mol. Biol. 348: 491-501.
Zampa A, Silvi S, Fabiani R, Morozzi G, Orpianesi C, Cresci A (2004). Effects of different digestible carbohydrates on bile acid metabolism and SCFA production by human gut micro-flora grown in an *in vitro* semi-continuous culture. Anaerobe 10: 19-26.