Pathol. Oncol. Res. (2011) 17:219–227
DOI 10.1007/s12253-010-9300-8

RESEARCH

Involvement of *c-Ski* Oncoprotein in Carcinogenesis of Cholangiocacinoma Induced by *Opisthorchis viverrini* and *N*-nitrosodimethylamine

Thidarut Boonmars · Zhiliang Wu · Sirintip Boonjaruspinyo · Anucha Puapairoj · Butsara Kaewsamut · Isao Nagano · Somchai Pinlaor · Puangrat Yongvanit · Orasa Wonkchalee · Amornrat Juasook · Pakkayanee Sudsarn · Tuanchai Srisawangwong

Received: 16 June 2009 / Accepted: 12 August 2010 / Published online: 18 September 2010

Abstract Opisthorchiasis is the major public health problem in the endemic areas of Thailand and Laos because *Opisthorchis viverrini* infection causes serious hepatobiliary diseases including CCA. The molecular mechanism of the CCA carcinogenesis induced by the infection remains obscure. To reveal the potential genes and signaling pathways to involve in the carcinogenesis, the present study investigated the expression of *c-Ski,* an oncogene, and two *TGF-β* signaling pathway relative genes, *TGF-β* and *Smad4*, during the development of CCA induced by *O. viverrini* infection in hamster model, and in human opisthorchiasis associated CCA. The results showed that the expression of *c-Ski* gene was greatly up-regulated during the carcinogenesis of CCA in hamster model. The overexpression of *c-Ski* was confirmed by immunohistological staining result which showed the increased expression of *c-Ski* protein in cytoplasm of the epithelial lining of hepatic bile ducts. Moreover, the immunohistological staining of the specimens of human opisthorchiasis associated CCA revealed the up-regulated expression of *c-Ski* and *Smad4* proteins in the cytoplasm of the epithelial lining of hepatic bile ducts and stomal fibrosis respectively. The expression of *TGF-β* and *Smad4* were up-regulated, which expression kinetics was time-dependent of CCA development. These results suggest that *c-Ski* is likely involved in the carcinogenesis of CCA induced by *O. viverrini* infection through regulating *TGF-β* signaling pathway.

Keywords Opisthorchiasis · *Opisthorchis viverrini* · *c-Ski* · *TGF-β* · CCA · Carcinogenesis

Abbreviations

CCA	Choangiocarcinoma
TGF-β	Transforming growth factor-*β*
NDMA	*N*-nitrosodimethylamine
Smad4	*SMAD* family member 4
c-Ski	*Ski* proto-oncogene

T. Boonmars (✉) · S. Boonjaruspinyo · S. Pinlaor · O. Wonkchalee · A. Juasook · P. Sudsarn · T. Srisawangwong
Department of Parasitology, Faculty of Medicine,
Khon Kaen University,
Khon Kaen 40002, Thailand
e-mail: bthida@kku.ac.th

T. Boonmars · S. Boonjaruspinyo · S. Pinlaor · P. Yongvanit
Liver Fluke and Cholangiocarcinoma Research Center,
Faculty of Medicine, Khon Kaen University,
Khon Kaen 40002, Thailand

Z. Wu · I. Nagano
Department of Parasitology,
Gifu University Graduate School of Medicine,
Yanagido1-1,
Gifu 501-1194, Japan

A. Puapairoj
Department of Pathology, Faculty of Medicine,
Khon Kaen University,
Khon Kaen 40002, Thailand

B. Kaewsamut
Northeast Laboratory Animal Center, Khon Kaen University,
Khon Kaen 40002, Thailand

P. Yongvanit
Department of Biochemistry, Faculty of Medicine,
Khon Kaen University,
Khon Kaen 40002, Thailand

Introduction

Opisthorchiasis, caused by a bile duct parasite, *Opisthorchis viverrini*, is mainly endemic in Southern Asia, including in Northeastern Thailand, Laos, Vietnam and Cambodia [1, 2]. The infection of this parasite has become major public concern because it is a significant risk factor for cholangiocarcinoma (CCA) development [1, 2]. Although CCA is a rare primary malignant epithelial liver tumor in the world, high incidence is observed in the endemic areas of *O. viverrini*, especially in Northern Thailand where is high endemic area of opisthorchiasis with the highest incidence rate of CCA in the world [1, 2]. The epidemiologic investigations and experimental animal researches have confirmed the definite correlation between occurrence of CCA and *O. viverrini* infection. The studies using hamster animal model revealed that infection caused chronic inflammation infiltration, epithelial desquamation, epithelial and adenomatous hyperplasia, goblet cell metaplasia, periductal fibrosis and granuloma formation [3]. Moreover, in hamster models, only infection or only *N*-nitrosodimethylamine (NDMA) administration dose not induce formation of CCA, but infection plus NDMA administration do causes the development of hamster CCA [4–6].

Little has been known on what kinds of genes or signaling pathways to be involved the carcinogenesis of the *O. viverrini* infection associated CCA. One of the candidate genes is *c-Ski*, which was first identified as the transforming protein of the avian Sloan-Kettering virus [7]. The oncogenic activity of *c-Ski* is primarily defined by its ability to promote anchorage-independent growth of chicken and quail embryo fibroblast when overexpressed [8], and by its roles in cell differentiation and transformation [9–13]. *c-Ski* is expressed in almost all adult and embryonic tissues but at low levels [7, 14, 15]. Up-regulation of *c-Ski* expression occurs during certain stages of embryonic development [16] and later in many human cancers [13, 17–19]. *C-Ski* protein levels and subcellular localization correlate with many human cancer and have been associated with clinical pathological parameters and tumor progression in several human malignancies [12, 18, 20]. Closely related to *c-Ski* is transforming growth factor beta (*TGF-β*) signaling pathway, which usually functions to suppress cellular differentiation, proliferation, and transformation [21]. As a negative regulator of the *TGF-β* signaling pathway, *c-Ski* is expected to be an important factor in carcinogenesis through suppressing *TGF-β* signaling pathway. Therefore, in the present study, we investigated the expression kinetics of *c-Ski* and its relative signaling pathway genes, *TGF-β* and *Smad4* during carcinogenesis of CCA induced by *O. viverrini* infection. The results suggested that *c-Ski*, as mediators in *TGF-β* signaling pathway, might be implicated in the carcinogenesis of *O. viverrini* infection associated CCA.

Materials and Methods

Parasites and Infection

O. viverrini metacercariae were isolated from naturally-infected cyprinoid fishes captured from a fresh water reservoir in an endemic area in Khon Kaen of Northeast Thailand using conventional method. The metacercariae, average size 201 μm×167 μm with oval excretory bladder containing a dense mass of dark granules, the brownish-yellow pigments scattered throughout the body, and oral and ventral sucker, were identified as the method described by Kaewkes[22].

Male Syrian hamsters, aged 6–8 weeks, from the Animal Unit, Faculty of Medicine, Khon Kaen University, were used in the present study. The protocol was approved by the Animal Ethics Committee of the Faculty of Medicine, Khon Kaen University, Thailand (Ethical Clearance No AEKKU0514.1.12/438).

In hamster animal models, only infection or only administration with NDMA do not induce the development of CCA, but infection plus administration of NDMA could induce the development of CCA. Therefore, total 60 hamsters were grouped into four groups: (1) normal group: 15 hamsters without any treatment; (2) infection group: 15 hamsters were orally infected with 50 metacercariae; (3) NDMA group: 15 hamsters were administrated with water containing12.5 ppm of NDMA (Wako, Japan) from 30 days after beginning of experiment; and (4) infection plus NDMA group: 15 hamsters were orally infected with 50 metacercariae, and then administrated with water containing12.5 ppm of NDMA from 30 day after infection. The livers of five hamsters at 30, 60 and 120 days p.i. were collected and subjected for the analysis of histopathology, genetic expression and immunohistochemistry.

Hamster Normal Liver and CCA Tissue

To collect CCA tissues for positive control, hamsters were infected with *O. viverrini* infected and then administrated with NDMA for 4-5 months to let CCA formation. Only CCA tissues were collected for RNA extraction as described below. Normal liver tissues were collected for negative control.

Surgical Human CCA Specimens

The paraffin embedded specimens of 15 cases of human CCA with opisthorchiasis history were obtained from the Liver Fluke and Cholangiocarcinoma Research Center,

Faculty of Medicine, Khon Kaen University, Thailand. All the 15 cases were from endemic area and were diagnosed as opisthorchiasis by observation of *Opisthorchis viverrini* egg in feces. Use of the specimens in the present experiments was approved by the Human Ethics Committee of the Khon Kaen University, (Ethical Clearance No HEKKU501153).

Light Microscopic Observation

Livers were fixed in 10% formalin and paraffin sections (6 μm thick) were prepared as conventional method. The sections were processed for hematoxylin and eosin (H&E) staining as described previously [23].

Total RNA Isolation and Reverse Transcription

Total RNA was extracted from the tissues (200 mg) at the hilar region of hamster livers from the four groups mentioned above (normal control, infection, NDMA and infection plus NDMA), using TRIZOL (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. In additional, the total RNA from liver tissues with normal bile duct and CCA tissues were isolated for negative and positive controls. The isolated RNA was treated with DNase (RQ1 RNase-Free DNase, Promega, Co., Madison, WI, USA). The treated RNA was extracted with phenol/chloroform, precipitated with ethanol, and dissolved in RNase-free water.

Reverse transcription was performed using M-MLV (Fermentas, Germany) according to the manufacturer's instructions. In brief, 20 μl reaction volume consisted of 3 μg of the sample RNA, 1 μl of 0.5 μg/μl Oligo (dT)15 (Amersham Phamacia Biotech, Inc., Piscataway, NJ, USA), 1 μl 10 mM deoxyribonucleotide triphosphate mix, 4 μl First-stand buffer, 1 μl 0.1 M dithiothreitol, 1 μl RNase inhibitor and one reverse transcriptase. The reaction mixture was incubated at 42°C for 60 min and then inactivated by heating at 70°C for 15 min.

Quantification by Real time PCR

The expression of *c-Ski, TGF-β* and *Smad4* were investigated and *G3PDH* (glyceraldehyde- 3-phosphate dehydrogenase) was used as an endogenous control. Primers were designed based on the published sequence in GenBank as follow: *c-Ski*: accession no: *AF435852*, ctgcgagtgagaaaga gacg/ttttcgtggctggatacaag; *TGF-β*: accession no: *AF046214*, gctggaagtggattcacga /cttgcgacccacgtagtaca; *Smad4*: accession no: *AF046214*, gacaaggtggggaaagtgaa/ ctccacagacgggcatagat; *G3PDH*: accession no: *M32599*, ggcattgtggaagggctcat/gacacattgggggtaggaacac. The specificity of these primers was confirmed by general PCR with the cDNA of liver tissue by observation of single band of each gene and without product with parasite cDNA.

Real-time PCR was performed to determine the expression levels of *c-Ski, TGF-β* and *Smad4* using a SYBR Premix Ex Taq Kit and Thermal Cycler Dice Real Time System (TAKARA BIO Inc., Ootsu, Shiga, Japan) according to manufacturer's instructions. In brief, 20 μl of reaction mixture consisted of 2 μl of the template (1:10 dilution), 10 μl of SYBR Premix Ex Taq, 0.8 μl of 5 μM of each primer and 6.4 μl of distilled water. PCR amplification was performed under one cycle pre-denature at 95°C for 10 s and 40 cycles amplification at 95°C for 5 s and 60°C for 30 s. Melting curve analysis was performed from 60°C to 95°C with 0.1°C/s temperature transition.

Specific external controls were established for the above target genes. The PCR fragment with specific primer pairs from each gene was cloned into a pT7Blue T-Vector (Novagen, Inc., Madison, WI, USA) and transformed into competent cells, *Escherichia coli* JM 109. Recombinant plasmid DNA was isolated from *E. coli* using a FlexiPrep Kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Ten-fold serial dilutions (10^1 to 10^7 copies/2 μl) of the plasmid DNA were used to generate standard curves for each gene.

The amounts of cDNA from different samples were normalized based on a quantification of the housekeeping gene *G3PDH*, and the gene expression levels were represented as the copy number of target gene/10^7 *G3PDH* copies. Values are expressed as the mean ± S.D. *P* values <0.05 were considered significant.

Immunohistochemical Staining for *c-Ski* and *Smad4* Proteins

Deparaffinized sections from not only the CCA tissues, but the non-cancerous livers were studied as well were processed for immunohistochemical staining. In brief, the sections were blocked with 5% skim milk in phosphate buffered saline (PBS), incubated with a 1:50 dilution of rabbit polyclonal antibodies against *c-Ski* or *Smad4* antigen (ZYMED Laboratories Inc., San Francisco, CA, USA) at 37°C for 1 h, and washed three times with in PBS. The second antibody was a 1:100 dilution of goat anti-rabbit IgG –HRP antibodies (ZYMED Laboratories Inc. San Francisco, CA, USA) with detection based on diaminobenzidene according to the manufacturer's instructions. The negative control was normal rabbit serum instead of the primary antibody.

Results

Gross and Histopathological Changes Associated with Hamster Opisthorchiasis

The gross appearance and histopathological changes of the hamster hepatobiliary system caused by *O. viverrini*

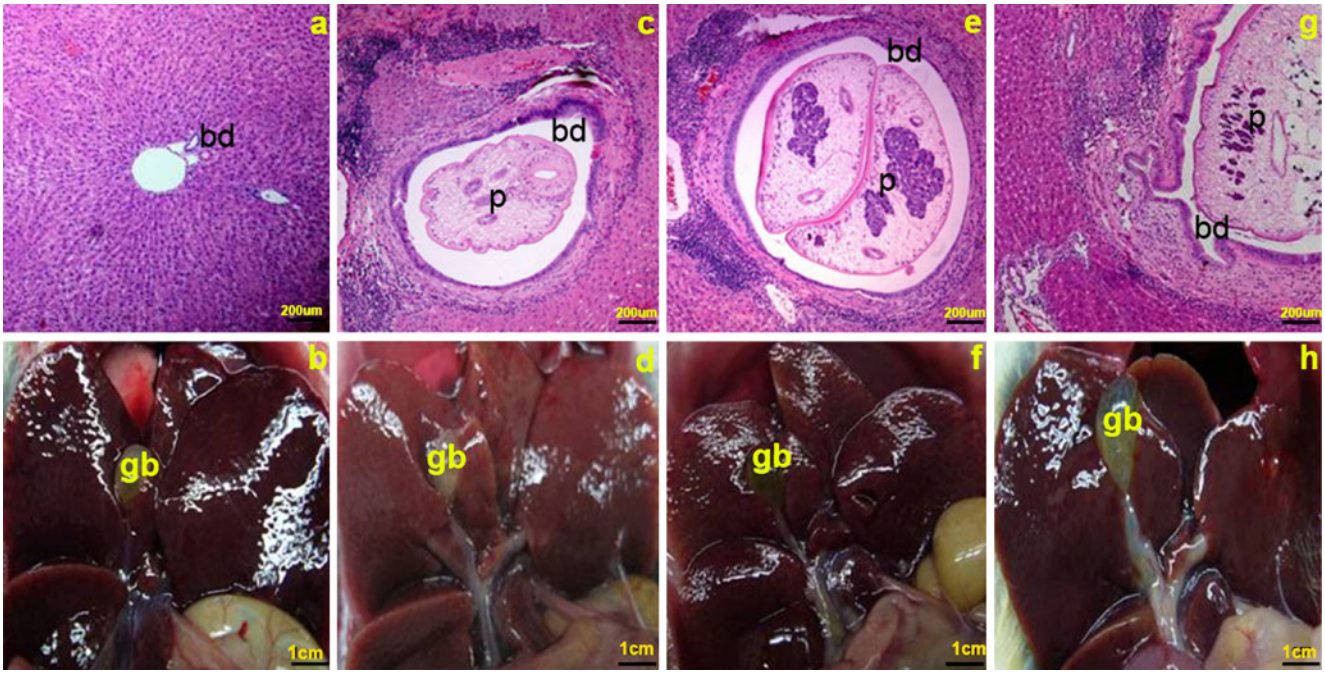

Fig. 1 Histopathological changes and gross anatomy of hamster with *Opisthorchis viverrini* infection alone at 0 day p.i. (**a** and **b**), 30 days p.i. (**c** and **d**), 60 days p.i. (**e** and **f**), and 120 days p.i. (**g** and **h**). *p* parasite adult; *bd* bile duct; *gb* gallbladder

infection were monitored at 0, 30, 60 and 120 days p.i. As shown in Fig. 1, the external bile ducts and gall bladder were gradually enlarged and became opaque by thickening of the wall with infection times (Fig. 1d, f, and h). The pathological features of the infected with *O. viverrini* group included an accumulation of inflammatory cells, mononuclear cells and eosinophils around the infected area. The most obvious histological changes observed at 60 and

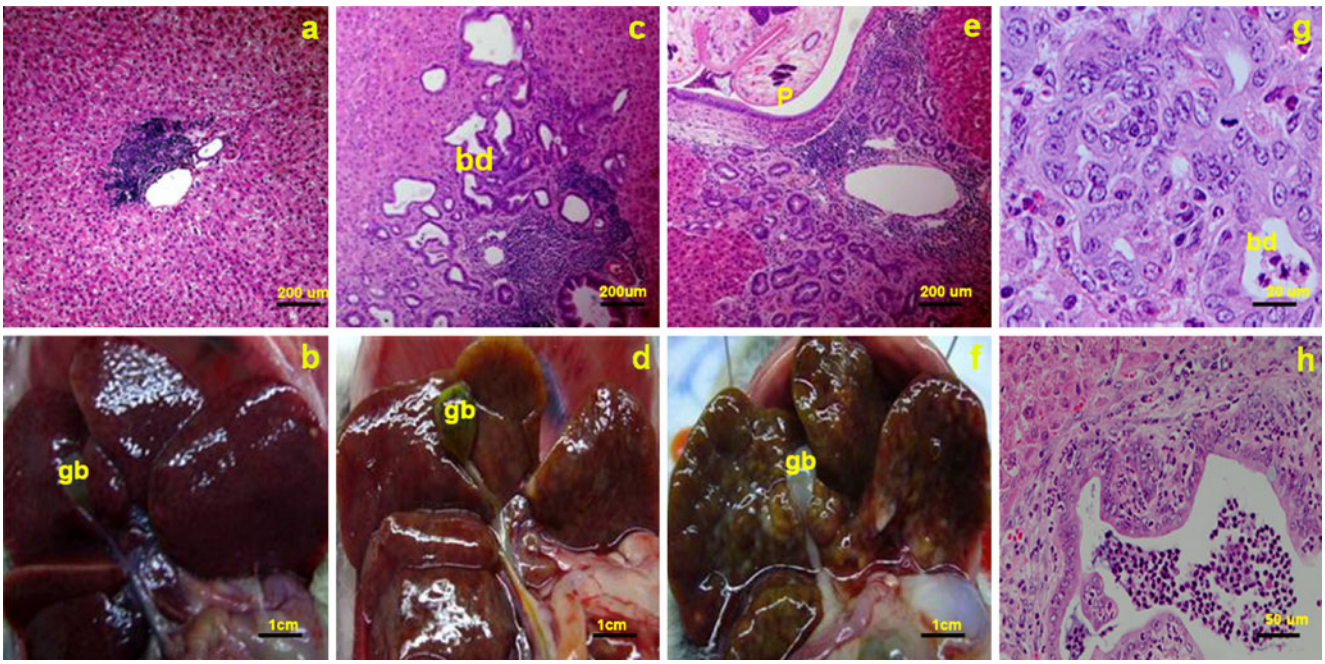

Fig. 2 Histopathological changes and gross anatomy of hamster with *N*-nitrosodimethylamine (NDMA) treatment alone at 60 days p.i. (**a** and **b**), and *Opisthorchis viverrini* infection plus NDMA treatment at 60 days p.i (**c** and **d**) and 120 days p.i. (**e**, **f**, **g** and **h**). Figure g and h showed malignancy dysplastic bile duct and cholangitis. *p* parasite adult; *bd* bile duct; *gb* gallbladder

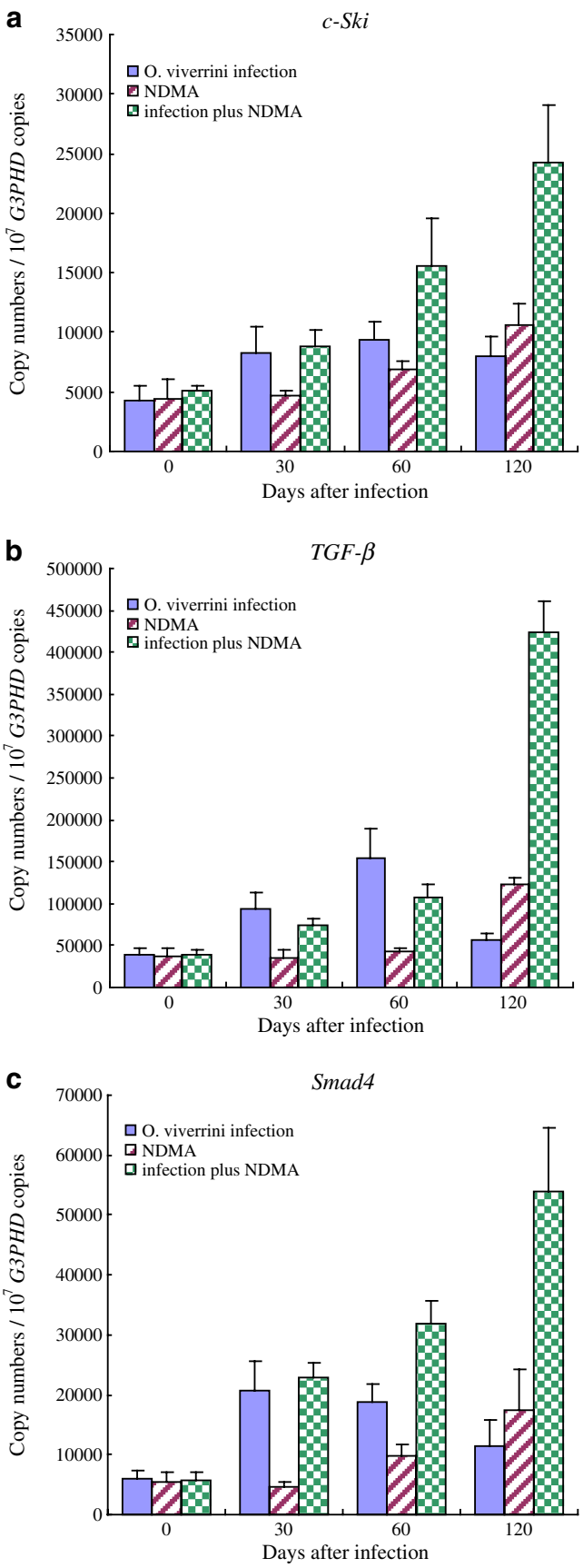

Fig. 3 The kinetics of expression levels of *c-Ski* (**a**), *TGF-* β(**b**) and *Smad4* (**c**) in the four groups (Normal control, infection, *N*-nitrosodimethylamine (NDMA) treatment, and infection plus NDMA). Total RNA was isolated from the hilar region of liver at various stages (0, 30, 60 and 120 days p.i.). The expression level of each gene was determined with real time PCR and was presented as copy numbers within 10^7 glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*) copies. The value was expressed as the mean ± S.D from five hamsters in each group

120 days p.i. were periductal fibrosis, ductal dilatation, hyperplastic changes and mononuclear cell infiltration with lymphoid aggregation (Fig. 1e and g).

Gross and Histopathological Changes of Hamster CCA Development

The gross appearance and histopathological changes of the hamster hepatobiliary system caused *O. viverrini* infection plus NDMA treatment were monitored at 0, 30, 60 and 120 days p.i. The results at 0 and 30 days p.i. were similar with those in Fig. 1a and b. As a comparison, Fig. 2a and b showed the results of NDMA group (treated with NDMA alone) at 120 days p.i. Compared results of Fig. 2a and b, the extrahepatic bile ducts and gall bladders in the group of infection plus NDMA treatment were enlarging and opaque with the time manner (Fig. 2d and f). Grossly, the liver surface was rough and abundant nodules were observed. The degree of opacity increased gradually and the liver's surface became nodular (Fig. 2d and f). Aggregation of active inflammatory cells surrounding the hepatic bile ducts and portal triad area was observed in all NDMA treatment group (Fig. 2a, c and e). In the group of infection plus NDMA treatment at day 60 and 120 there was pronounced aggregation of active inflammatory cells surrounding the hepatic bile ducts and portal triad area. Hyperplastic bile duct cells (excess of bile duct formation) was observed in the group of infection plus NDMA treatment at day 60 days p.i. (Fig. 2c), but not in the group of NDMA treatment alone (Fig. 2a). In the group of infection plus NDMA treatment at 120 days p.i., thickening of the hepatic bile ducts, small bile duct proliferation and inflammatory cells were observed, and inflammatory cells were observed in the bile duct canals and in the bile duct epithelial tissue, which is a pathology well known in acute cholangitis with abscess (Fig. 2h) and dysplasia of bile ducts (Fig. 2g).

Expression Kinetics of *c-Ski*, *TGF-*β and *Smad4* in Hamster Opisthorchiasis and CCA Development

The expression kinetics of *c-Ski*, *TGF-*β and *Smad4* in 3 hamster groups (infection, NDMA, and infection plus NDMA) at 0, 30, 60 and 120 days p.i. was investigated. As shown in Fig. 3a, the expression level of *c-Ski*

persistently increased after infection plus NDMA treatment, from 5,104 copies in 10^7 *G3PDH* copies before infection, to 7,854 at 30 days p.i., 15,574 at 60 days p.i., and 24,253 copies at 120 days p.i. NDMA treatment alone caused a slight increase in the expression of *c-Ski*. The expression level increased to 6,903 and 10,620 copies at 60 and 120 days p.i., respectively, but significantly lower than in infection plus NDMA group ($P<0.01$). Infection only caused some degree increase of *c-Ski* expression, from 4,292 copies before infection, to 8,316 at 30 days p.i., 9,336 at 60 days p.i., and 8,024 copies at 120 days p.i.

Similar expression pattern as *c-Ski* was observed in *TGF-β* and *Smad4*. As shown in Fig. 3b, the expression of *TGF-β* greatly increased with infection time in infection plus NDMA group, from 38,975 copies before infection to 424,120 copies at 120 days p.i., nearly 11 folds. Infection group showed a temporary increase in expression of *TGF-β*, increasing from 39,405 copies before infection to the peak (155,160 copies) at 60 days p.i. and then returning to 56,833 copies at 120 days p.i. In NDMA group, there was not significant increase in the expression before 60 days p.i. ($P>0.05$), but an increase of expression was observed at 120 days p.i. The expression kinetics of *Smad4* was quite similar with *TGF-β*, as shown in Fig. 3c.

The Expression of *c-Ski*, *TGF-β* and *Smad4* in CCA Tissues

To confirm whether the changed expression of the genes is from CCA tissue or not, CCA was induced by infection plus NDMA treatment and CCA tissue only was isolated. The expression of *c-Ski*, *TGF-β* and *Smad4* in the CCA tissue was determined and compared with the expression in normal liver. As shown in Fig. 4, the expression of *c-Ski* and *TGF-β* were greatly increased in CCA tissues, about 6.4 and 18.6 folds higher than in normal liver, respectively. The expression of *Smad4* in CCA tissue was 2.6 folds higher than the expression in normal liver.

Immunohistochemical Staining for *c-Ski* in Hamster Tissues

To determine the in situ distribution of the up-regulated expression of *c-Ski* protein, paraffin sections of tissues (hilar region of liver) from the infection group and infection plus NDMA group at 30, 60 and 120 days p.i. were immunostained with an anti-*c-Ski* antibody. As shown in Fig. 5, strong positive staining for *c-Ski* protein was observed in the cytoplasm of epithelium lining of the hepatic bile duct in the infection plus NDMA group at 60 (Fig. 5c) and 120 days p.i. (Fig. 5d). The staining of *c-Ski* was undetectable in the tissues of infection group at 30, 60 and 120 days p.i. (only the result at 60 days p.i. is presented in Fig. 5b). Staining specificity was confirmed by the absence of staining when using normal rabbit serum (Fig. 5a).

Immunohistochemical Staining for *c-Ski* and *Smad4* in Human Opisthorchiasis-Associated CCA

To observe whether there is expression change of *c-Ski* and *Smad4* in human opisthorchiasis-associated CCA, the sections of liver tissues, obtained from surgical specimens of 15 CCA cases who were from *O. viverrini* endemic area and have opisthorchiasis history, were immunostainined with anti *c-Ski* and anti- *Smad4* antibodies. As shown in Fig. 6, strong staining for *c-Ski* protein was observed in malignancy bile duct areas (Fig. 6b and c). The positive staining for *Smad4* protein was observed at the stromal fibroblasts, connective tissue which located among the malignancy bile duct, and some area of epithelial lining (Fig. 6d, e and f). Staining specificity was confirmed by the absence of staining when using normal rabbit serum (Fig. 6a).

Discussion

In the present study, at the first time we confirmed the overexpression of *c-Ski* during the carcinogenesis of CCA

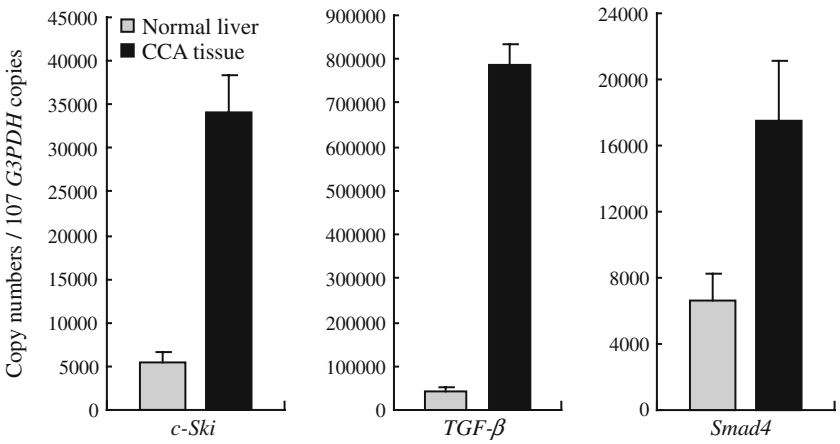

Fig. 4 The expression levels of *c-Ski*, *TGF-β* and *Smad4* in normal liver and *Opisthorchis viverrini* infection-induced cholangiocarcinoma (CCA) tissues. Total RNA was isolated from the hilar region of normal liver and the CCA tissues. The expression level of each gene was determined with real time PCR, and was presented as copy numbers within 10^7 glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*) copies. The value was expressed as the mean ± S.D from five hamsters

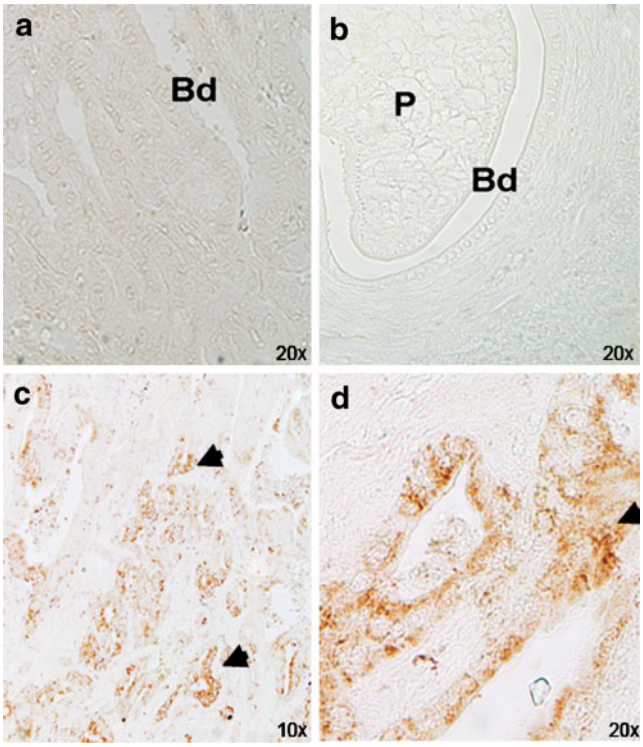

Fig. 5 Immunohistochemical staining for *c-Ski*. Deparaffinized sections of hamster liver infected with *Opisthorchis viverrini* alone at 60 days p.i (**b**), infection plus NDMA treatment at 60 days p.i (**c**) and 120 days p.i (**d**), were stained with anti-*c-Ski* antibody. Panel a showed a negative control staining with normal rabbit serum. The brown color indicates the positive reaction, showed by arrow. *P* parasite adult; *Bd* bile duct

induced by *O. viverrini* infection. The overexpression was indicated by (1) up-regulated expression of *c-Ski* gene in *O. viverrini* infected plus NDMA treated hamster model, (2) greatly up-regulated expression of *c-Ski* gene in CCA tissues, (3) increased expression of *c-Ski* protein in the cytoplasm of epithelium lining of the hepatic bile duct in the opisthorchiasis associated CCA of hamster, and (4) increased expression of *c-Ski* protein in the human opisthorchiasis CCA. Meanwhile, the expression kinetics of *TGF-β* and *Smad4* genes was corresponding to the process of carcinogenesis of CCA in hamster model. These results suggest that *c-Ski* oncoprotein may involve in the carcinogenesis induced by *O. viverrini* infection, likely through regulating *TGF-β* signaling pathway.

Hamster is only animal model for studying opisthorchiasis associated CCA, which has been widely used now. Infection or NDMA treatment alone does not induce formation of CCA in the model, while infection plus NDMA treatment could induce carcinogenesis, leading to the formation of CCA [4–6]. The present study further confirmed the usefulness of this animal model. The gross observation and histopathological results indicated pathological features of CCA carcinogenesis. After infection followed with NDMA treatment, the extrahepatic bile ducts and gall bladders became enlarging and opaque with the time manner, and hyperplastic bile duct cells were observed. At late stage (120 days p.i.), there was the thickening of the hepatic bile ducts, small bile duct proliferation, cholangitis with abscess, and dysplasia of bile ducts, which were not observed in infection or NDMA treatment alone.

Corresponding to these pathological changes, the expression of *c-Ski* gene was up-regulated. The highest expression

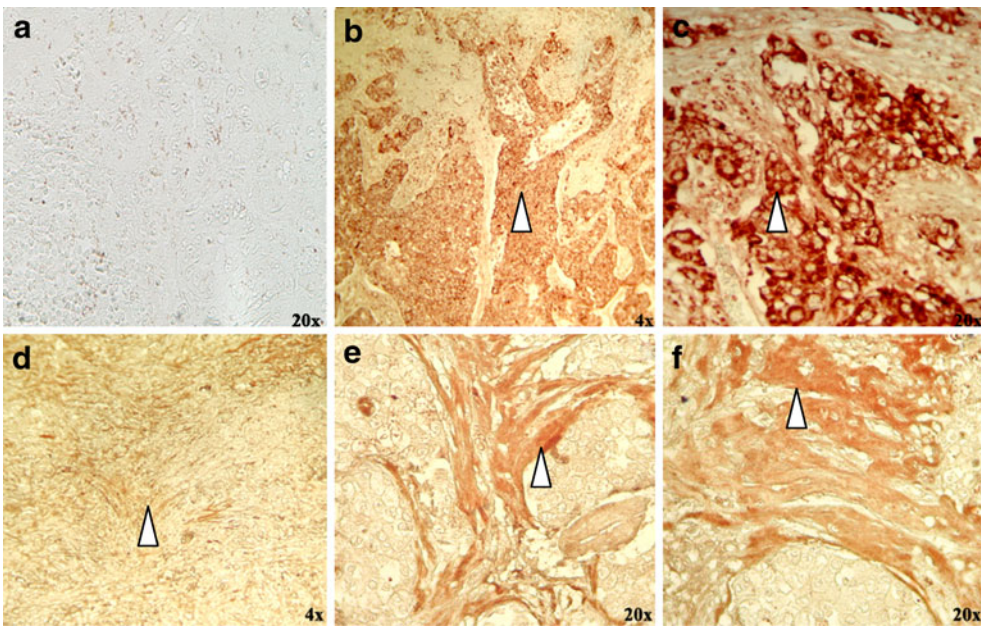

Fig. 6 Immunohistochemical staining of human opisthorchiasis-associated cholangiocarcinoma (CCA) for *c-Ski* (**b** and **c**) and *Smad4* (**d**, **e** and **f**). Deparaffinized sections of human CCA tissues were stained with anti-*c-Ski* antibody (**b** and **c**), and anti-*Smad4* antibody (**d**, **e** and **f**). Same kinds of sections were stained with normal rabbit serum as negative control (**a**). The *brown color* indicates the positive reaction, showed by arrow

level was observed in the liver tissues at 60 days p.i. in infection plus NDMA hamsters. A great overexpression was observed in the isolated CCA tissues induced by infection. Moreover, immunostaining with anti *c-Ski* antibody indicated that the increased expression of *c-Ski* protein was limited in the cytoplasm of epithelium lining of the hepatic bile duct in the infection plus NDMA treated hamsters, and also strong staining was observed in tissue of human opisthorchiasis associated CCA. These results also support the hypothesis that *c-Ski* is synthesized from cholangiocyte which is increased with the time manner of CCA development.

So far the most important function described for *c-Ski* is to negatively regulate *TGF-β* signaling pathway [24]. *TGF-β* signaling pathway usually functions to suppress cellular proliferation and transformation, known as a signaling to suppress tumor development. *C-Ski* represses *TGF-β* signaling pathway by modifying *Smad* activity, displacing coactivators from *Smad* complexes, and preventing nuclear translocation of *Smad* proteins [12, 25–27]. Obviously the repression of *TGF-β* signaling pathway contributes to carcinogenesis. The overexpression of *c-Ski* has been observed in many types of cancer [13, 18, 19, 28]. Down-regulation of *c-Ski* protein levels by antisense vectors or RNAi technology [12] inhibits cancer cell growth and clonogenicity, and restores *TGF-β*-mediated growth inhibition. These results highlight the critical role of *c-Ski* in cancer proliferation, and suggest a potential to break cancer proliferation by targeting *c-Ski*. The overexpression of *c-Ski* in the present study suggests that *c-Ski* is involved in the carcinogenesis of CCA induced by *O. viverrini*. The results also provide a proposal to therapy of CCA by targeting *c-Ski*.

TGF-β is a ubiquitous cytokine that plays role in cell differentiation, proliferation, apoptosis and morphogenesis [21]. It is expressed in kupffer cells, endothelial cells and hepatic stellate cells, and not expressed in normal cholangiocytes but activated cholangiocytes [29, 30]. The up-regulation of *TGF-β* expression has been observed in many types of cancer including cholangiocarcinoma [30–32]. Its functions in regulating cell growth and the markedly increased expression in cancer make this important cytokine to be considered as predictive biomarkers for progressive tumorigenesis, molecular targets for prevention and treatment of cancer, and metastasis [33, 34]. Our present results showed that *TGF-β* is highly increased in hamster CCA development induced by *O. viverrini* infection plus NDMA treatment. The increased expression was time-dependent. The expression of *TGF-β* at 120 days p.i. in *O. viverrini* infection plus NDMA group was 10.9 fold higher than in normal group. Moreover, the expression *TGF-β* in CCA tissues was 18.6 fold higher than in normal liver. Although infection alone caused temporary increase in *TGF-β* expression at 60 days p.i., the expression decreased at 120 days p.i. and its level was much lower than in infection plus NDMA group. These results suggest a potential of the *TGF-β* as biomarker for *O. viverrini* infection-induced CCA.

TGF-β signaling-induced transactivation of various reporter genes by its interaction with *Smad4* proteins [16, 25, 35–37]. *Smad4* belongs to the *smad* family of proteins, reported to be a tumor suppressor [37]. We found except the high expression of *Smad4* in *O. viverrini* infection plus NDMA group, infection alone also caused up-regulation of the expression, especially at 30 and 60 days p.i. The increased expression may contribute to inflammatory cells, as reported by Kim et al. [39]. More important is that the expression of *Smad4* protein expressed was increased in the stomal fibrosis and connective tissue among malignancy bile duct, and the epithelial lining of human opisthorchiasis-associated CCA tissues (Fig. 6d, e and f), which agree with the report by Kang et al. [40] and Liu et al. [41] that *Smad4* expressed in the cytoplasm of the epithelial lining (fibroblasts) of bile duct of human intrahepatic CCA.

In conclusion, present study indicated the overexpression of *c-Ski* in hamster and human opisthorchiasis-associated CCA. The results suggest that *c-Ski* may play an important role during carcinogenesis of CCA induced by *O. viverrini* infection, through regulating *TGF-β* signaling pathway. In the future, the basic knowledge obtained in this work may contribute to development of new tools for diagnosis, prognosis determination, therapeutic treatment, testing of new drugs, and research on the biology of the development and progression of this disease.

Acknowledgments This research was supported by the Research Strengthening Grant from National Center for Genetic Engineering and Biotechnology (BIOTEC), Graduate School of Medicine Facuty, Graduate School of Khon Kaen University, National Science and Technology Development Agency (NSTDA), Grant-in-Aid for Scientific Research (21590463) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Heiwa Nakajima Foundation which supported Dr. Thidarut Boonmars to stay in Gifu University Graduate School of Medicine in Japan for carrying out a part of experiments of the present research.

References

1. IARC (1994) Infection with liver flukes (*Opisthorchis viverrini, Opisthorchis felineus* and *Clonorchis sinensis*). IARC Monogr Eval Carcinog Risk Chem Hum 61:121–175
2. Sithithaworn P, Haswell-Elkins M (2003) Epidemiology of *Opisthorchis viverrini*. Acta Trop 88:187–194
3. Sripa B (2003) Pathobiology of opisthorchiasis: an update. Acta Trop 88:209–220
4. Thamavit W, Kongkanuntn R, Tiwawech D, Moore MA (1987) Level of Opisthorchis infestation and carcinogen dose-dependence of cholangiocarcinoma induction in Syrian golden hamsters. Virchows Arch B Cell Pathol Incl Mol Pathol 54:52–58

5. Thamavit W, Pairojkul C, Tiwawech D et al (1993) Promotion of cholangiocarcinogenesis in the hamster liver by bile duct ligation after dimethylnitrosamine initiation. Carcinogenesis 14:2415–2417
6. Thamavit W, Pairojkul C, Tiwawech D, Shirai T, Ito N (1994) Lack of promoting effect of proline on bile duct cancer development in dimethylnitrosamine-initiated hamster livers. Teratog Carcinog Mutagen 14:169–174
7. Li Y, Turck CM, Teumer JK, Stavnezer E (1986) Unique sequence, ski, in Sloan-Kettering avian retroviruses with properties of a new cell-derived oncogene. J Virol 57:1065–1072
8. Colmenares C, Stavnezer E (1989) The ski oncogene induces muscle differentiation in quail embryo cells. Cell 59:293–303
9. Fumagalli S, Doneda L, Nomura N, Larizza L (1993) Expression of the *c-Ski* proto-oncogene in human melanoma cell lines. Melanoma Res 3:23–27
10. Pelzer T, Lyons GE, Kim S, Moreadith RW (1996) Cloning and characterization of the murine homolog of the sno proto-oncogene reveals a novel splice variant. Dev Dyn 205:114–125
11. Atanasoski S, Notterpek L, Lee HY et al (2004) The protooncogene Ski controls Schwann cell proliferation and myelination. Neuron 43:499–511
12. Reed JA, Bales E, Xu W, Okan NA, Bandyopadhyay D, Medrano EE (2001) Cytoplasmic localization of the oncogenic protein Ski in human cutaneous melanomas in vivo: functional implications for transforming growth factor beta signaling. Cancer Res 61:8074–8078
13. Reed JA, Lin Q, Chen D, Mian IS, Medrano EE (2005) SKI pathways inducing progression of human melanoma. Cancer Metastasis Rev 24:265–272
14. Markowitz S, Wang J, Myeroff L et al (1995) Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. Science 268:1336–1338
15. Miyaki M, Iijima T, Konishi M et al (1999) Higher frequency of *Smad*4 gene mutation in human colorectal cancer with distant metastasis. Oncogene 18:3098–3103
16. Namciu S, Lyons GE, Micales BK et al (1995) Enhanced expression of mouse *c-Ski* accompanies terminal skeletal muscle differentiation in vivo and in vitro. Dev Dyn 204:291–300
17. Zhang F, Lundin M, Ristimäki A et al (2003) Ski-related novel protein N (SnoN), a negative controller of transforming growth factor-beta signaling, is a prognostic marker in estrogen receptor-positive breast carcinomas. Cancer Res 63:5005–5010
18. Fukuchi M, Nakajima M, Fukai Y et al (2004) Increased expression of *c-Ski* as a co-repressor in transforming growth factor-beta signaling correlates with progression of esophageal squamous cell carcinoma. Int J Cancer 108:818–824
19. Ritter M, Kattmann D, Teichler S et al (2006) Inhibition of retinoic acid receptor signaling by Ski in acute myeloid leukemia. Leukemia 20:437–443
20. Bravou V, Antonacopoulou A, Papadaki H et al (2009) TGF-beta repressors SnoN and Ski are implicated in human colorectal carcinogenesis. Cell Oncol 31:41–51
21. Shi Y, Massague J (2003) Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113:685–700
22. Kaewkes S (2003) Taxonomy and biology of liver flukes. Acta Trop 88:177–186
23. Boonmars T, Srisawangwong T, Srirach P et al (2007) Apoptosis-related gene expressions in hamsters re-infected with *Opisthorchis viverrini* and re-treated with praziquantel. Parasitol Res 102:57–62
24. Luo K (2004) Ski and SnoN: negative regulators of TGF-beta signaling. Curr Opin Genet Dev 14:65–70
25. Akiyoshi S, Inoue H, Hanai J et al (1999) *c-Ski* acts as a transcriptional co-repressor in transforming growth factor-beta signaling through interaction with smads. J Biol Chem 274:35269–35277
26. Prunier C, Pessah M, Ferrand N et al (2003) The oncoprotein Ski acts as an antagonist of transforming growth factor-beta signaling by suppressing *Smad2* phosphorylation. J Biol Chem 278:26249–26257
27. Suzuki H, Yagi K, Kondo M et al (2004) *c-Ski* inhibits the TGF-beta signaling pathway through stabilization of inactive *Smad* complexes on *Smad*- binding elements. Oncogene 23:5068–5076
28. Zhang B, Halder SK, Zhang S, Datta PK (2009) Targeting transforming growth factor-beta signaling in liver metastasis of colon cancer. Cancer Lett 277:114–120
29. Patsenker E, Popov Y, Stickel F et al (2008) Inhibition of integrin alphavbeta6 on cholangiocytes blocks transforming growth factor-beta activation and retards biliary fibrosis progression. Gastroenterology 135:660–670
30. Dumont N, Arteaga CL (2000) Transforming growth factor-β and breast cancer: tumor promoting effects of transforming growth factor-β. Breast Cancer Res 2:125–132
31. Milani S, Herbst H, Schuppan D, Stein H, Surrenti C (1991) Transforming growth factors beta 1 and beta 2 are differentially expressed in fibrotic liver disease. Am J Pathol 139:1221–1229
32. Levy L, Hill CS (2006) Alterations in components of the *TGF-β* superfamily signaling pathways in human cancer. Cytokine Growth Factor Rev 17:41–58
33. Dranoff G (2004) Cytokines in cancer pathogenesis and cancer therapy. Nat Rev Cancer 4:11–22
34. Jakowlew SB (2006) Transforming growth factor-beta in cancer and metastasis. Cancer Metastasis Rev 25:435–457
35. Laiho M, DeCaprio JA, Ludlow JW, Livingston DM, Massagué J (1990) Growth inhibition by TGF-beta linked to suppression of retinoblastoma protein phosphorylation. Cell 62:175–185
36. Hannon GJ, Beach D (1994) p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. Nature 371:257–261
37. Iavarone A, Massague J (1999) E2F and histone deacetylase mediate transforming growth factor beta repression of cdc25A during keratinocyte cell cycle arrest. Mol Cell Biol 19:916–940
38. Jang CW, Chen CH, Chen CC et al (2002) TGF-beta induces apoptosis through *Smad*-mediated expression of DAP-kinase. Nat Cell Biol 4:51–58
39. Kim BG, Li C, Qiao W et al (2006) *Smad4* signalling in T cells is required for suppression of gastrointestinal cancer. Nature 441:1015–1019
40. Kang YK, Kim WH, Jang JJ (2002) Expression of G1-S modulators (p53, p16, p27, cyclin D1, Rb) and *Smad4*/Dpc4 in intrahepatic cholangiocarcinoma. Hum Pathol 33:877–883
41. Liu Y, Wang LF, Zou HF, Song XY, Xu HF, Lin P, Zheng HH, Yu XG (2006) Expression and location of *Smad2*, 4 mRNAs during and after liver fibrogenesis of rats. World J Gastroenterol 14;12 (10):1577–1582