RESEARCH COMMUNICATION

# Cholangiocarcinoma in Experimental Hamsters with Long-standing *Opisthorchis viverrini* Infection

**Nopparat Songserm[1,4], Jiraporn Prasongwattana[1], Paiboon Sithithaworn[1,3], Banchob Sripa[2,3], Vichit Pipitgool[1]**

**Abstract**

**Liver fluke infection of *Opisthorchis viverrini* (*O. viverrini*) is closely associated with several hepatobiliary diseases including cholangiocarcinoma (CCA), but no reports have described these diseases in chronic and long-standing experimental opisthorchiasis in hamsters more than 10 months of age. A longer period of infection could induce different pathological lesions. To prove the hypothesis, we therefore sequentially investigated histological changes of the hepatobiliary system in 4 groups of hamster: *O. viverrini* infection (OV group) for up to 20 months, *O. viverrini* infection combined with short-term DMN (OV+DMN group) until 7 months, long-term treatment with DMN (DMN group) to 7 months and normal control group for up to 20 months. Pathological changes in hamsters of the OV group gradually increased. Induction of CCA in this study was apparent with all three protocols. Importantly, this is the first report of CCA-induction in hamsters solely with long-term opisthorchiasis for up to 20 months. Although the histopathology of CCA in the OV group showed some differences in appearance from the OV+DMN and DMN groups, overall, *O. viverrini* itself can really induce CCA. In addition, this study confirms the previous studies both *in vitro* and *in vivo* on of effects of parasites and their metabolic products inducing cell proliferation, resulting in cholangiocarcinogenesis.**

**Key Words:** Cholangiocarcinoma - hamster model - opisthorchiasis - carcinogenicity

*Asian Pacific J Cancer Prev,* **10**, 299-302

## Introduction

Liver cancer is the fifth most common cancer in the world; there are 315,000 new cases of CCA in the world each year (Parkin et al., 1993). Most of them are found in the people of northeast Thailand, where is the high prevalence of *O. viverrini* infection (Kurathong et al., 1985; Elkins et al., 1990; Watanapa, 1996). Opisthorchiasis occur in people who consumed the improper cooked and fermented fresh water cyprinoids fish. CCA has been developed in some of them after several years of infection when they reach the middle age. It is proposed that CCA may be developed in people with long-standing infection of *O. viverrini.*

Experimental infections of *O. viverrini* have been studied in hamster to understand how CCA could be developed (Bhamarapravati et al., 1978; Thamavit et al., 1978; Thamavit et al., 1987; Thamavit et al., 1993; Thamavit et al., 1994; Sripa and Kaewkes, 2000a; Sripa and Kaewkes, 2002). The first report proposed that *O. viverrini* infection is promoter and carcinogen (dimethylnitrosamine, DMN) is initiator of carcinogenesis (Bhamarapravati et al., 1978). There are several studies have been reported the factors concerning CCA induction in hamster model such as endogenous nitrosation (Satarug et al., 1998), metabolic products of worm (Thuwajit et al., 2004) and DNA damage in chronic and repeated infection (Pinlaor et al., 2004a; Pinlaor et al., 2004b) but until now no reports about long-term infection in hamster to see whether those factors could really concern in CCA induction. Moreover, those studies have not been done in hamster more than 10 months of infection. It is questionable that the longer period of infection could induce any differences in pathological appearances. To prove the hypothesis of *O. viverrini* itself, endogenous nitrosation together with long-standing infection of *O. viverrini* could induce chronic opisthorchiasis and CCA. Therefore, our study designs the long-term opisthorchiasis compare to the induced CCA in hamster and investigates them according to gross and histological features of hepatobiliary system.

## Materials and Methods

*Experimental hamsters*

Randomly bred male golden hamsters, 6-8 weeks old that reared and supplied by the Animals Care Unit, Faculty of Medicine, Khon Kaen University were used in the

*[1]Department of Parasitology, [2]Department of Pathology, [3]Liver fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen, [4]Department of Community Health, Faculty of Science, Ubon Ratchathani Rajabhat University, Ubon Ratchathani, Thailand *For Correspondence: songsermn@hotmail.com*

study. The protocol was approved by the Animal Ethic Committee of the Faculty of Medicine, Khon Kaen University.

*O. viverrini metacercaria and their induction to hamster*

Cyprinoid fish from endemic area of Khon Kaen Province were collected, minced and digested with pepsin. Metacercaria of *O. viverrini* were selected and counted under the stereomicroscope and after that they were fed on hamster via gastric tube.

*Carcinogen administration*

DMN (Sigma, USA) was diluted in distilled water under biohazard hood. The final concentration of solution was 12.5 ppm, kept in a dark container, and transferred to brown bottles used only for hamsters with DMN administration in a separate room.

Short-term administration: 12.5 ppm of DMN solution was supplied to hamster for 10 weeks, starting from the time of *O. viverrini* infection. After that they received normal drinking water until sacrificed. Long-term administration: hamsters received the same concentration of DMN solution from the beginning until the day of sacrifice.

*Study design*

1. OV group: hamsters infected with 50 metacercaria of *O. viverrini* were divided into seven groups. Each group (4-6 hamsters/group) was sacrificed according to the schedule of 4th, 8th, 12th, 14th, 16th, 18th and 20th month after infection (see Figure 1).

2. OV+DMN group: hamsters treated with 50 metacercaria of *O. viverrini* combine with 12.5 ppm of DMN for 10 weeks. There are 7 groups of hamsters; each group (4-6 hamsters/group) was sacrificed according to the schedule of 1st, 2nd, 3rd, 4th, 5th, 6th and 7th month after treatment.

3. DMN group: hamsters administrated with continuously long-term of DMN were divided into 2 groups. Each group (4-6 hamsters/group) was sacrificed according to the schedule of 4th and 7th month after treatment.

4. Control group: hamsters received neither metacercaria nor DMN were reared in the same condition as the experimental hamsters. There are 4 groups of hamsters; each group (4-6 hamsters/group) was sacrificed according to the schedule of 4th, 8th, 12th and 20th month.

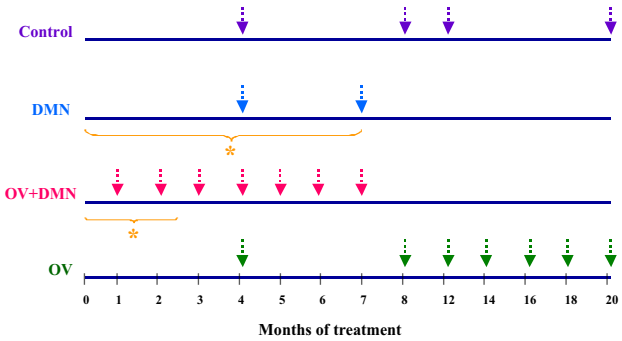


**Figure 1. Experimental Procedure for CCA Induction in Hamsters.** DMN, dimethylnitrosamine; OV, *Opisthorchis viverrini*; *, period of DMN treatment; arrows, sacrifice points

Blood was drawn from the orbital plexus of hamsters. After that, hamsters of each group were sacrificed under deep anesthesia, blood was also drawn directly from the heart and the liver was removed. The livers were examined for gross pathology and histopathology. Four lobes of the liver were studied for hepatic nodules, hepatic fibrosis and tumor mass. Later the livers were fixed in 10% buffer formalin, serially sliced in a 4-5 mm thickness, embedded in paraffin. Sections were cut at 4 µm thickness and stained with hematoxylin-eosin. Histopathological features of bile duct epithelium (e.g. inflammatory cell infiltration, bile duct dilatation, periductal fibrosis, bile duct epithelial hyperplasia, cholangiofibrosis, goblet cell metaplasia, dysplasia and CCA) and hepatic parenchyma (e.g. cirrhosis and hepatocellular carcinoma, HCC) were carefully investigated under light microscope.

## Results

Pathological changes in hamsters with *O. viverrini* infection alone gradually increased over time (Figure 2). Hepatic nodules and hemorrhagic spots in 8th, 12th, 14th and 18th month post-infection (p.i.). However, hamsters of this group no showed tumor mass. Hamsters of OV+DMN group showed tumor mass since 3rd month and severity was continuously increased. Hamsters of DMN group showed tumor mass in all hamsters of 7th month, but not in hamsters of 4th month after continuously long-term administration of DMN.

Histopathological findings of all experimental groups

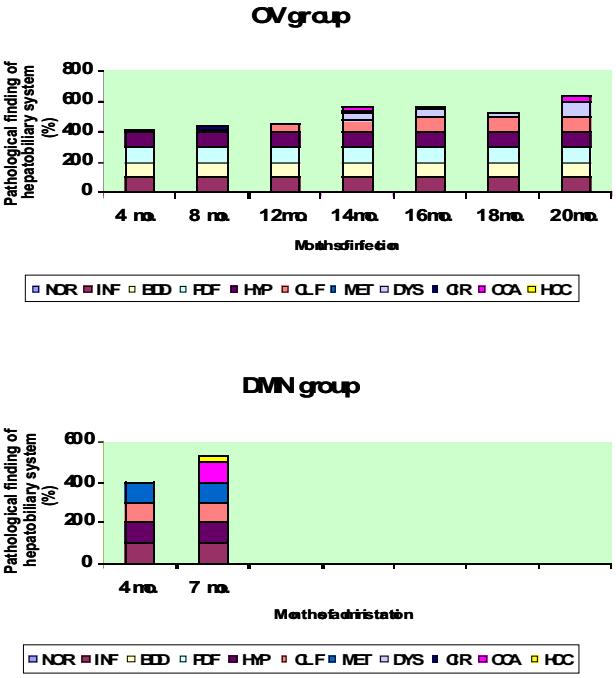


**Figure 2. Pathological Findings for the OV and DMN Experimental Groups According to the Duration of Infection/Treatment**. NOR, normal appearance; INF, inflammatory cell infiltration; BDD, bile duct dilatation; PDF, periductal fibrosis; HYP, bile duct epithelial hyperplasia; CLF, cholangiofibrosis; MET, goblet cell metaplasia; DYS, dysplasia; CIR, cirrhosis; CCA, cholangiocarcinoma; and HCC, hepatocellular carcinoma

showed identical features of bile duct epithelium such as inflammatory cells infiltration, periductal fibrosis, bile duct epithelial hyperplasia, cholangiofibrosis. Cirrhosis, histopathological feature of hepatic parenchyma was found only in OV group. Importantly, dysplasia was also found only in OV group as early as 14th month p.i. , while goblet cell metaplasia was showed in only group of carcinogenic-induced hamsters (OV+DMN and DMN groups).

Although no gross appearance of tumor mass in OV group, histopathological features of CCA was found in 2 of 9 hamsters in 14th and 20th month p.i., respectively. All hamsters of OV+DMN group from 3rd month p.i. and all hamsters of DMN group in 7th month after long-term treatment showed CCA. In addition, 1 of 5 hamsters of DMN group in 7th month showed CCA together with HCC. Interestingly, histopathology of CCA in OV group showed some appearances different from OV+DMN and DMN groups.

Intensity of malignant foci of CCA was quantitative analysis by histological findings. Approximately 1-10 foci of 0.06 cm per section were found in only right lobe liver of OV group at 14th and 20th month. For OV+DMN group, the malignant foci were found throughout the liver, mostly in right, left, medium and caudal lobes, respectively as early as 3rd month post treatment. Distributions of malignant foci with size of 0.1-1.53 cm were found more than 10 foci per section. Similarly, malignant foci with 0.125-1 cm were showed in all hamsters of DMN group at 7th month after treatment, but mostly distribute in left, right, medium and caudal lobes, respectively. In addition, malignant foci were not found in normal control hamsters. However, the distributions of malignant foci were not statistically significant among 3 liver lobes, except caudal lobe in carcinogenic-induced groups (OV+DMN and DMN).

## Discussion

In the present study CCA induction was successful with all three protocols. Until now, no previous reports on CCA induction in hamsters by long-term infection of *O. viverrini* alone for up to 10 months of infection and by continuous administration of DMN alone more than 10 weeks. Although *O. viverrini*-infected hamsters have been reported at 9th month p.i., not showed at other time point of experiment (Thamavit et al., 1993; Sithithaworn et al., 2002).

In this study, not all hamsters with *O. viverrini* infection alone develop to the CCA at the same time. Histopathological appearance of CCA was found in half number of hamsters at 14th and 20th month p.i. Indeed, due to cancer induction may be responsible to individual difference of genetics that code for DNA repairing, immune response and inflammation (Jaiswal et al., 2000). Although some hamsters did not showed histopathology of CCA, all of them showed cholangiofibrosis, goblet cell metaplasia and dysplasia. According to these criteria of histopathology, they were identified to be precancerous lesion of CCA (Thamavit et al., 1993). Differently, the incidence of CCA in the group of carcinogenic-induced hamsters (OV+DMN and DMN groups) was 100% at the same time. All hamsters of OV+DMN group from 3rd month and all of DMN group in 7th month have developed the CCA. These results may derive from the different mechanisms of carcinogenesis among the two groups of CCA-induced hamsters, with or without DMN. More detail is needed to be further study.

There are many cancers are associated with infectious agents, e.g., virus (hepatitis B and C), bacteria (*Helicobactor pylori*), and parasite (*O. viverrini*), which can promote carcinogenesis through the induction of chronic inflammatory states (Parkin, 2006). According to CCA, endogenous nitrosation, disability of DNA repairing system and decrease in ability of tumor suppressor function are the major causes of carcinogenesis (Satarug et al., 1998; Pinlaor et al., 2004a). In the same way with our experiment, *O. viverrini* alone can induce CCA in long-standing infection. This occurrence may derive from the actions of oxygen radicals, e.g., nitric oxide (NO) released from cytokines that can induce oxidative DNA damage to the infected biliary epithelium (Pinlaor et al., 2003; Pinlaor et al., 2004a). Furthermore, repeated infections with *O. viverrini* contribute to rising DNA damage through increased nitric oxide synthase expression in biliary epithelium (Pinlaor et al., 2004b). In summation, NO not only induces DNA damage but has been reported to mediate DNA repair inhibition (Jaiswal et al., 2000; Jaiswal et al., 2001).

At present, the diagnosis of CCA can be elusive; it is often not made until advanced disease is present and at a stage when a curative surgical resection is not feasible. Attempts to solve these problems have been done in many aspects of CCA both in man and experimental animals. The hamster has been described as the suitable experimental animals due to the natural pathway of disease as same as in man. The *O. viverrini*-infected hamster was used to examine the pathology of hepatobiliary system; early pathological changes consisted of inflammation, periductal fibrosis, and proliferative responses, including epithelial hyperplasia, goblet cell metaplasia, and adenomatous hyperplasia, may represent predisposing lesions that enhance susceptibility of DNA to carcinogens (Bhamarapravati et al., 1978; Sripa and Kaewkes, 2000a). In chronic infection, periportal and periductal fibrosis were the most prominent histological features. However, the inflammatory responses are less severe in chronic infection than the acute one suggesting that immunomodulation may occur (Sripa and Kaewkes, 2000a).

There are several researches have been studied on pathogenesis of liver fluke-induced CCA, both worm's own and its metabolic products. Mechanical injury from fluke's suckers hook on the biliary epithelium for feeding and moving, resulting in tissue damage even early in infection (Bhamarapravati et al., 1978). As the fluke matures, the lesion becomes more obvious and ulcerated. The metabolic products of liver fluke were secreted or excreted from the tegument and excretory openings into bile or culture medium in vitro (Wongratanacheewin et al., 1988; Sripa and Kaewkes, 2000b). The metabolic products themselves may be toxic to or interact with the

biliary epithelium for inducing host immune responses (Harinasuta et al., 1984). There are some studies have been attempted to describe how metabolic products of liver fluke induced cell proliferation, but most of them were performed in vitro, e.g., mouse fibroblast (Thuwajit et al., 2004) and human CCA cell line (Sripa et al., 2005). For *in vivo* study, bile duct epithelial hyperplasia, as a form of cell proliferation was found in hamsters with opisthorchiasis (Bhamarapravati et al., 1978; Sripa and Kaewkes, 2000a), with presentation of *O. viverrini* antigens in periductal of bile duct, macrophages, epithelioid cells, and giant cells of the granuloma, as detected by immunohistochemistry (Sripa and Kaewkes, 2000a).

Although *in vivo* study in hamsters indicated that *O. viverrini* itself and its metabolic products can induce cell proliferation, we here showed for the first time that long term exposure to the parastite alone can really induce CCA.

## References


Bhamarapravati N, Thamavit W, Vajrasthira, S (1978). Liver changes in hamsters infected with a liver fluke of man, *Opisthorchis viverrini*. *Am J Trop Med Hyg*, **27**, 787-94.

Elkins DB, Haswell-Elkins MR, Mairiang E, et al (1990). A high frequency of hepatobiliary disease and suspected cholangiocarcinoma associated with heavy *Opisthorchis viverrini* infection in a small community in north-east Thailand. *Trans R Soc Trop Med Hyg*, **84**, 715-9.

Harinasuta T, Riganti M, Bunnag D (1984). *Opisthorchis viverrini* infection: pathogenesis and clinical features. *Arzneimittelforschung*, **34**, 1167-9.

Jaiswal M, LaRusso NF, Burgart LJ, Gores GJ (2000). Inflammatory cytokines induce DNA damage and inhibit DNA repair in cholangiocarcinoma cells by a nitric oxide-dependent mechanism. *Cancer Res*, **60**, 184-90.

Jaiswal M, LaRusso NF, Shapiro RA, Billiar TR, Gores GJ (2001). Nitric oxide-mediated inhibition of DNA repair potentiates oxidative DNA damage in cholangiocytes. *Gastroenterology*, **120**, 190-9.

Kurathong S, Lerdverasirikul P, Wongpaitoon V, et al (1985). Opisthorchis viverrini infection and cholangiocarcinoma. A prospective, case-controlled study. *Gastroenterology*, **89**, 151-6.

Parkin DM (2006). The global health burden of infection-associated cancers in the year 2002. *Int J Cancer*, **118**, 3030-44.

Parkin DM, Oshima H, Srivatanakul P, Vatanasapt V (1993). Cholangiocarcinoma: epidemiology, mechanism of carcinogenesis and prevention. *Cancer Epidemiol Biomarkers Prev*, **2**, 537-44.

Pinlaor S, Hiraku Y, Ma N, et al (2004a). Mechanism of NO-mediated oxidative and nitrative DNA damage in hamsters infected with *Opisthorchis viverrini*: a model of inflammation-mediated carcinogenesis. *Nitric Oxide*, **11**, 175-83.

Pinlaor S, Ma N, Hiraku Y, et al (2004b). Repeated infection with *Opisthorchis viverrini* induces accumulation of 8-nitroguanine and 8-oxo-7,8-dihydro-2'-deoxyguanine in the bile duct of hamsters via inducible nitric oxide synthase. *Carcinogenesis*, **25**, 1535-42.

Pinlaor S, Yongvanit P, Hiraku Y, et al (2003). 8-nitroguanine formation in the liver of hamsters infected with *Opisthorchis viverrini*. *Biochem Biophys Res Commun*, **309**, 567-71.

Satarug S, Haswell-Elkins MR, Sithithaworn P, et al (1998). Relationships between the synthesis of N-nitrosodimethylamine and immune responses to chronic infection with the carcinogenic parasite, *Opisthorchis viverrini*, in men. *Carcinogenesis*, **19**, 485-91.

Sithithaworn P, Ando K, Limviroj W, et al (2002). Expression of tenascin in bile duct cancer of hamster liver by combined treatment of dimethylnitrosamine with *Opisthorchis viverrini* infections. *J Helminthol*, **76**, 261-8.

Sripa B, Kaewkes S (2000a). Localisation of parasite antigens and inflammatory responses in experimental opisthorchiasis. *Int J Parasitol*, **30**, 735-40.

Sripa B, Kaewkes S (2000b). Relationship between parasite-specific antibody responses and intensity of *Opisthorchis viverrini* infection in hamsters. *Parasite Immunol*, **22**, 139-45.

Sripa B, Kaewkes S (2002). Gall bladder and extrahepatic bile duct changes in *Opisthorchis viverrini*-infected hamsters. *Acta Trop*, **83**, 29-36.

Sripa B, Leungwattanawanit S, Nitta T, et al (2005). Establishment and characterization of an opisthorchiasis-associated cholangiocarcinoma cell line (KKU-100). *World J Gastroenterol*, **11**, 3392-7.

Thamavit W, Bhamarapravati N, Sahaphong S, Vajrasthira S, Angsubhakorn S (1978). Effects of dimethylnitrosamine on induction of cholangiocarcinoma in *Opisthorchis viverrini*-infected Syrian golden hamsters. *Cancer Res*, **38**, 4634-9.

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**, 52-8.

Thamavit W, Pairojkul C, Tiwawech D, Itoh M, Shirai T, Ito N (1993). Promotion of cholangiocarcinogenesis in the hamster liver by bile duct ligation after dimethylnitrosamine initiation. *Carcinogenesis*, **14**, 2415-7.

Thamavit W, Pairojkul C, Tiwawech D, Shirai T, Ito N (1994). Strong promoting effect of *Opisthorchis viverrini* infection on dimethylnitrosamine-initiated hamster liver. *Cancer Lett*, **78**, 121-5.

Thuwajit C, Thuwajit P, Kaewkes S, et al (2004). Increased cell proliferation of mouse fibroblast NIH-3T3 in vitro induced by excretory/secretory product(s) from *Opisthorchis viverrini*. *Parasitology*, **129**, 455-64.

Watanapa P (1996). Cholangiocarcinoma in patients with opisthorchiasis. *Br J Surg*, **83**, 1062-64.

Wongratanacheewin S, Bunnag D, Vaeusorn N, Sirisinha S (1988). Characterization of humoral immune response in the serum and bile of patients with opisthorchiasis and its application in immunodiagnosis. *Am J Trop Med Hyg*, **38**, 356-62.