RESEARCH COMMUNICATION

Ultrasonography as a Tool for Monitoring the Development and Progression of Cholangiocarcinoma in *Opisthorchis viverrini*/ Dimethylnitrosamine-Induced Hamsters

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Abstract

Cholangiocarcinoma (CCA) is the most common cancer in northeastern Thailand. At present, effective diagnosis of CCA either in humans or animals is not available. Monitoring the development and progression of CCA in animal models is essential for research and development of new promising chemotherapeutics. Ultrasonography has been widely used for screening of bile duct obstruction in CCA patients. In this study, we preliminarily investigated the applicability of ultrasonography to monitor the development and progression of CCA in Syrian golden hamsters (n=8) induced by *Opisthorchis viverrini* (OV)/dimethylnitrosamine (DMN) administration. Ultrasonography and histopathological examination of hamsters was performed at week 0, 20, 24 and 28 of OV infection or at the start of water/Tween-80 administration to controls. The ultrasonographic images of liver parenchyma and gallbladders of OV/DMN-induced CCA hamsters showed sediments in gallbladder, thickening of gallbladder wall, and hypoechogenicity of liver parenchyma cells. The ultrasonographic images of liver tissues were found to correlate well with histopathological examination. Although ultrasonography does not directly detect the occurrence of CCA, it reflects the thickening of bile ducts and abnormality of liver tissues. It may be applied as a reliable tool for monitoring the development and progression of CCA in animal models in research and development of new promising chemotherapeutics for CCA.

Keywords: Cholangiocarcinoma - ultrasonography - diagnosis - hamster - *Opisthorchis viverrini* - dimethylnitrosamine

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Introduction

Cholangiocarcinoma (CCA) is a slow-growing ductal adenocarcinoma of the liver, with relatively rare incidence of about 3-7% of malignant liver tumors (Minami and Kudo, 2010). This type of cancer is an important public health problem in several parts of Southeast Asia, particularly the northeastern region of Thailand (Sripa et al., 2010). The major cause of CCA in Thailand is consumption of improper cooked and fermented fresh water cyprinoids fish called 'Pla-ra' or 'Pla-som', which contains *Opisthorchis viverrini* (OV) and nitrosamine (Sripa et al., 2007). Lack of effective diagnostic tool and chemotherapeutics are major constraints for controlling this type of cancer. Chemotherapy and radiotherapy are only effective in patients with early stage, whilst the majority of patients come to receive treatment when cancer progresses to advanced stage (Fava and Lorenzini, 2002). Early diagnosis is therefore crucial for effective treatment of CCA (Zografos et al., 2011). Attempts have been made both in human and experimental animals to search for diagnostic tools for early detection and monitoring the progression of CCA, but each has shortcoming and limitation. These include the use of serum tumor markers (Ramage et al., 1995; Bjornsson et al., 1999; Xu et al., 2008; Tao et al., 2010; Silsirivanit et al., 2011; Sinakos et al., 2011), computed thermography (CT) scan (Nesbit, 1988), magnetic resonance imaging (MRI) (Yasutake et al., 1988; Manfredi et al., 2004; Vanderveen and Hussain, 2004), and ultrasonography (Karstrup, 1988; Nesbit, 1988; Colli et al., 1998; Tillich et al., 1998; Freeny, 1999). Recent advances in digital technologies have resulted in remarkable developments in the field of imaging modalities. CT scan and MRI are effective but are too expensive for routine clinical and experimental applications (Ustundag and Bayraktar, 2008). The simple, non-invasive and cost effective diagnosis by abdominal ultrasonography, although provides low sensitivity results, it is a useful tool to rule out liver diseases due to other causes (Bloom et al., 1999). In addition, it provides high

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level of community acceptance for screening of CCA in populations at risk in endemic areas of OV infection (Mairiang et al., 2011).

With respect to research and development of new promising chemotherapeutics for CCA, validity of animal models which closely mimic the pathogenicity of human CCA is a pre-requisite step. Experimental infection of OV with the carcinogen dimethyl nitrosamine (DMN) in hamsters has been described as the suitable experimental model due to its similarity of natural pathway of disease pathogenesis with that in humans (Bhamarapravati et al., 1978; Sripa and Kaewkes 2002). In all cases, the development and progression of CCA in these animals before or after treatment of test compounds/plant extracts can only be confirmed by histopathological examination of liver and gallbladder at autopsy (Thamavit et al., 1978). This may obscure or result in misinterpretation of therapeutic efficacy of the test substances, since the occurrence and stage of CCA development cannot be ensured prior to testing. The aim of the study was to preliminarily investigate the applicability of ultrasonography in detecting the development and monitoring the progression of CCA in hamsters following induction of CCA by OV and DMN (OV/DMN-induced CCA hamsters).

Materials and Methods

Induction of CCA in hamsters

A total of 16 (8 males and 8 females) Syrian golden hamsters, aged 6-8 weeks and weighting 105-120 g used in the study were purchased from The National Laboratory Animal Centre of Thailand. They were housed under standard conditions, and fed with a stock diet and water *ad libitum*. Approval of the study protocol was obtained from the Ethics Committee for Research in Animals, Thammasat University, Thailand. Cyprinoid fished were obtained from OV endemic areas in Khon Kaen Province, northeast of Thailand. The fishes were minced and digested with pepsin. Metacercariae of OV were selected and counted under light microscope. Development of CCA was induced in 8 hamsters (4 males and 4 females) by initial feeding of animals (through gastric gavage) with 50 metacercariae of OV, followed four weeks later by drinking water containing 12.5 *ppm* of dimethylnitrosamine (DMN: Sigma-Aldrich Inc., St. Louis, MO, USA) for eight weeks (Tesana et al. 2007). Control group (4 males, 4 females) received a mixture of water and Tween-80 (Sigma-Aldrich Inc., St. Louis, MO, USA) during the same period.

Ultrasonography and histopathological examination:

Ultrasonography and histopathological examination were performed in hamsters in both groups at week-0 (before OV infection or the start of administration of water/Tween-80), and thereafter at week 20, 24 and 28 (1 male and 1 female for each time point in each group). Ultrasonography (Logic P6, GE Healthcare Re-imagined, Solingen, Germany) was applied to detect the development and progression of CCA in hamsters. Before application, animals were fasted for three hours prior to anesthesization with isofurane (Minrad Inc., NY, USA). The development and progression of CCA was classified based on the ultrasonographic images into four grades as presented in Table 1.

Histopathology of the livers, bile ducts and gallbladders were examined after sacrification of all hamsters under deep anesthesia with ether. The livers were examined for gross pathology and histopathology. The livers were thereafter, fixed in 10% buffer formalin, serially sliced in about 5 mm thickness and embedded in paraffin. Sections were cut at about 5 μm thickness and stained with hematoxylin-eosin (Chaimuangraj et al., 2003). Histopathological features of bile duct epithelium were investigated under light microscope. The scheme of experimental design is shown in Table 2.

Table 1. Grading of CCA Pathology by Ultrasonography

CCA Grade	Bile duct	Liver parenchyma
0	Normal	Normal
1	Thickening	Mild
2	Thickening	Moderate
3	Thickening	Severe

Results

The development and progression of CCA was monitored by ultrasonography and histopathological examination at 0, 20, 24 and 28 weeks after treatment initiation in both control (water/Tween-80) and experimental (OV metacercariae) groups. Results obtained from ultrasonography and histopathological examination in both groups at each time point were found to be in good agreement. In the experimental group, abnormal changes in liver tissues from sacrificed hamsters showed development of tumor and pus in liver that corresponded with ultrasonographic results (hypoechoic nodule foci). The movement of leaf-shape organism, anticipating to be OV, was observed occasionally in gallbladder of the OV/DMN-induced CCA hamsters. Histopathological examination at autopsy at all time points (week 0, 20, 24 and 28) in OV/DMN-induced hamsters

Table 2. The Scheme of Experimental Design. X Symbol Indicates Ultrasonographic and Histopathological (autopsy) Investigations

	Control group (water+Tween-80) N=8								Experimental group (OV/DMN) N=8							
	Male (n=4)				Female (n=4)				Male (n=4)				Female (n=4)			
Time	No.1	No.2	No.3	No.4	No.5	No.6	No.7	No.8	No.1	No.2	No.3	No.4	No.5	No.6	No.7	No.8
Week 0	X				X				X				X			
Week 20		X				X				X				X		
Week 24			X				X				X				X	
Week 28				X				X				X				X

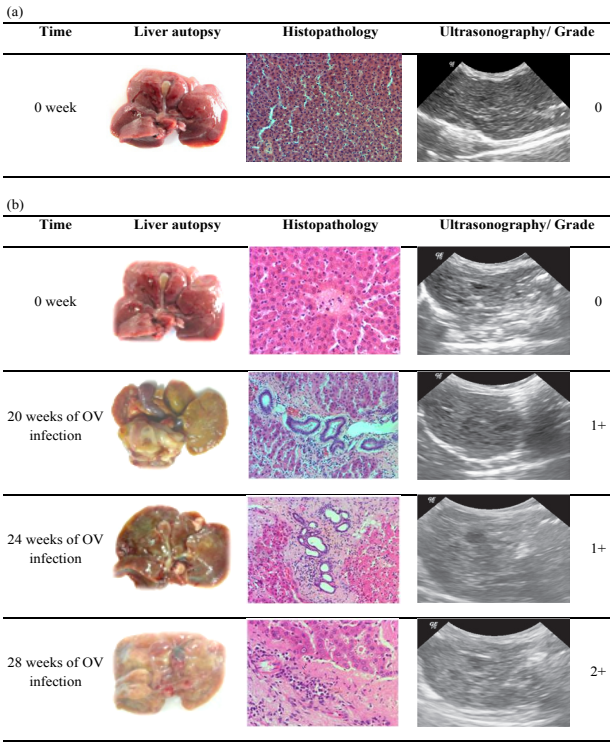

Figure 1. Representative Ultrasonographic Images and Histopathological Examinations of the Livers of (a) Control and (b) CCA-induced CCA hamsters observed at Time of Liver Autopsy.

revealed small foci and well-differentiated tubular adenocarcinoma throughout the liver mass that correlated with ultrasonographic image. Based on ultrasonographic results, none of the control hamsters developed CCA (CCA grade 0) or any other abnormality during the investigation period. Liver and gallbladders of all OV/DMN-induced CCA hamsters however, showed significant progression in the intensity of ultrasonographic images starting from week 20 (grade 1+), week 2 (grade 1+) to week 28 (grade 2+) after OV infection (Figure 1). The ultrasonographic images of the livers of the hamsters before OV infection, as well as those of the control hamsters showed uniformly hypoechoic with a coarse echotexture liver parenchyma. Twenty weeks post-infection, thickening of gallbladder wall with small amount of sediment and non-homogenous liver parenchyma was observed. Heterogenous and diffused hypoechogenicity of liver parenchyma was founded at week 24 post-infection. Progression of liver parenchymal changes with diffused hyperechoic with multiple hypoechoic nodules foci of liver parenchyma was found at week 28. All OV/DMN-induced CCA hamsters died at 28 weeks post-infection.

For all ages, adenocarcinoma was the most common histologic type, and more than one half of all tumours occurred in the colon, roughly one third of which was found in the right colon. There was no appreciable variation of tumour location between both age groups (Table 1). Mucin-producing and advanced-grade tumor were 2 times higher in proportion in the younger patients ($p < 0.001$). Advanced stage (III and IV) at diagnosis was observed in a slightly higher proportion in the younger (61%) than in the older (49%) group (Table 1).

Discussion

The Syrian hamster model closely mimics the histopathological lesions of CCA in humans and therefore, has been used as an animal model for CCA (Pairojkul et al, 1991). It was proposed that OV infection is promoter and the carcinogen DMN is initiator of carcinogenesis (Bhamarapravati et al., 1978). The severity of cholangiocellular lesions including pre-neoplastic cholagiofibrosis and CCA depend on both the dose of DMN and numbers of OV metacercariae infected (Thamavit et al., 1987). Our study was the first report demonstrating the applicability of ultrasonography in detecting the development and monitoring progression of CCA in QV/DMN-induced CCA hamsters. Abdominal ultrasonography has been widely used in human for diagnosis (screening), prognosis (staging) and monitoring progression of hepatocellular diseases including CCA (Bloom et al., 1999). In addition, it is also useful in disease surveillance following tumor ablation (Choi et al., 2008). Results showed that ultrasonography can be applied in research and development of new promising chemotherapeutics for CCA, as a reliable tool to detect and monitor the development and progression of CCA in animal models after treatment with candidate compounds/plant extracts. Ultrasonographic images of the livers of hamsters both in the control and QV/DMN-induced CCA group corresponded well with the gross morphology and histopathology of the livers obtained at autopsy (Figure 1). Histopathological findings of the group treated with OV/DMN showed inflammatory cell infiltration, periductal fibrosis, bile duct epithelial hyperplasia and cholangiofibrosis of bile ducts. The malignant foci were observed throughout the liver, with approximately 10 μm in size. The bile ducts were dilated due to the obstruction by tumors. Ultrasonography does not directly detect the occurrence/progression of CCA; however, it reflects the thickening of bile ducts and progression of abnormality of liver parenchyma and bile duct from normal (pre-infection) to moderate changes (24 and 28 weeks post-infection) (Figure 1). The relationship between intensity of OV infection and hepatobiliary disease detected by ultrasonography has previously been demonstrated (Mairiang et al, 1992). For the diagnosis of liver tumors in animals, contrast-enhanced ultrasonography with sonazoid has been applied for characterization of canine focal liver lesions (Nakamura et al., 2010). Although the sensitivity and specificity of ultrasonography is low, it is very useful to rule out gall stone obstruction and preoperative of hilar CCA (Nesbit, 1988). Ultrasonographic findings, inevitably requires confirmation by histopathological investigation.

In all studies investigating the candidate compounds or extracts of medicinal plants, the occurrence of CCA development in OV/DMN-induced CCA hamsters could not be ensured at the time when the test compounds/plant extracts were initially given. Rather, confirmation of the development of CCA was only possible when animals died by histopathological examination of liver tissues at autopsy. In such cases, interpretation or conclusion on anti-CCA activities of the test compounds/plant extracts

may not be reliable, since the absence or reduction of severity of CCA may not genuinely indicate their therapeutic efficacy. In contrast, ultrasonography, apart from its simplicity and non-invasiveness features, it also improves the accuracy of result interpretation, as it ensures the development/occurrence of CCA in animal enrolled in the experiment before treatment initiation with real-time monitoring of tumor progression all along the course of treatment.

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