Pathol. Oncol. Res. (2013) 19:55–62
DOI 10.1007/s12253-012-9557-1

RESEARCH

Immunosuppressive Prednisolone Enhances Early Cholangiocarcinoma in Syrian Hamsters with Liver Fluke Infection and Administration of *N*-nitrosodimethylamine

Amornrat Juasook · Thidarut Boonmars · Zhiliang Wu · Watcharin Loilome · Kulathida Veteewuthacharn · Nissana Namwat · Pakkayanee Sudsarn · Orasa Wonkchalee · Pranee Sriraj · Ratchadawan Aukkanimart

Received: 1 December 2011 / Accepted: 23 July 2012 / Published online: 1 August 2012
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Abstract Chronic infection with *Opisthorchis viverrini* for many years has been associated with the development of hepatobiliary diseases including cholangiocarcinoma. It is well known that inflammation is a key component of the tumor microenvironment, and that chronic inflammation plays an important role in tumorigenesis. Therefore, in this study cholangiocarcinogenesis was induced in Syrian hamsters in order to observe the cancer-related inflammation. The Syrian hamsters were divided into 5 groups: uninfected controls; normal Syrian hamsters infected with *O. viverrini* (OV); immunosuppressed Syrian hamsters infected with *O. viverrini* (OV*is*); normal Syrian hamsters infected with *O. viverrini* and administered *N*-nitrosodimethylamine (CCA); and immunosuppressed Syrian hamsters infected with *O. viverrini* and administered *N*-nitrosodimethylamine (CCA*is*). Syrian hamster livers were later observed for gross pathology and histopathological changes; COX2 was analyzed by immunohistochemical staining. We found a decreased number of inflammatory cells surrounding the hepatic bile duct in the OV*is* group, but not in the OV and CCA*is* groups. However, in the CCA*is* group (with suppressed immunity) early appearance and greater severity of cholangiocarcinoma were observed; gross pathological examination revealed many cancer nodularities on the liver surface, and histopathological studies showed the presence of cancer cells, findings which correlated with the predominant expression of COX2. The present study suggests that host immune responses are intended to ameliorate pathology, and they are also crucially associated with pathogenesis in *O. viverrini* infection; the unbalancing of host immunity may enhance cancer-related inflammation.

Keywords Liver fluke · Cholangiocarcinogenesis · Immunosuppressed · Chronic inflammation · Histopathology

A. Juasook · T. Boonmars (✉) · P. Sudsarn · O. Wonkchalee · P. Sriraj · R. Aukkanimart
Department of Parasitology, Faculty of Medicine,
Khon Kaen University,
Khon Kaen 40002, Thailand
e-mail: boonmars@yahoo.com

T. Boonmars
e-mail: bthida@kku.ac.th

A. Juasook · T. Boonmars · W. Loilome · K. Veteewuthacharn · N. Namwat · P. Sudsarn · O. Wonkchalee · P. Sriraj · R. Aukkanimart
Liver Fluke and Cholangiocarcinoma Research Center,
Khon Kaen University,
Khon Kaen 40002, Thailand

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

W. Loilome · K. Veteewuthacharn · N. Namwat
Department of Biochemistry, Faculty of Medicine,
Khon Kaen University,
Khon Kaen 40002, Thailand

Introduction

Opisthorchis viverrini is one of the risk factors for cholangiocarcinoma (CCA) development [1, 2] and is endemic

throughout Southeast Asia, including parts of Thailand, Laos, Vietnam and Cambodia [2]. CCA is a critical public health problem in northeastern Thailand. The disease constitutes approximately 50 % of all liver cancers in Thailand, and approximately 90 % of all liver cancers in northeast Thailand. *O. viverrini* infection induces CCA, which results in chronic inflammation through a combination of mechanical damage, parasite secretions and immunopathology [1]. At the early stages of infection, liver changes are due to the inflammatory response (eosinophils, monocytes and neutrophils) around the juvenile flukes in the intrahepatic bile ducts [3]. The virulence of the disease also involves the host's own immune response, such as cytokine expression and resultant free radicals [4]. Host immune responses can induce infiltration of the inflammatory cells surrounding the hepatic bile ducts by reactive oxygen species (ROS) and reactive nitrogen species (RNS) [5, 6]. ROS not only destroys parasites but also damages host tissues including DNA molecules, resulting in DNA fragmentation and cell apoptosis [7] . Moreover, RNS, which is produced during inflammation, may play a role in the initiation and subsequent modulation stage of CCA development by DNA mutation, leading to cancer development [4, 8]. However, several authors have suggested that the immunopathological process may contribute to the hepatobiliary inflammation and damage. Thus, chronic infection with *O. viverrini* for many years has been associated with several hepatobiliary diseases [3], which are in turn associated with the development of hepatobiliary cancer and cholangiocarcinoma [1]. Based on many research reports on a variety of cancers, inflammation is a key component of the tumor microenvironment; chronic inflammation has been demonstrated to play important roles in tumorigenesis, tumor progression, and metastasis [9, 10].

Therefore, in this study cholangiocarcinogenesis in immunosuppressed Syrian hamsters was observed to clarify the cancer-related inflammation. Immunity in Syrian hamsters was suppressed by administration of prednisolone in order to investigate the pathogenesis due to the inflammatory response in opisthorchiasis-induced cholangiocarcinoma. Syrian hamster livers were subsequently examined for gross pathological and histopathological changes, and immunohistochemistry via COX2 staining.

Materials and Methods

Animals

Syrian hamsters, 6 to 8 weeks old, from the Animal Unit, Faculty of Medicine, Khon Kaen University, were divided into five groups (20 Syrian hamsters per group): uninfected controls; normal Syrian hamsters infected with *O. viverrini* (OV); immunosuppressed Syrian hamsters infected with *O. viverrini* (OV*is*); normal Syrian hamsters infected with *O. viverrini* and administered *N*-nitrosodimethylamine (CCA); and immunosuppressed Syrian hamsters infected with *O. viverrini* and administered *N*-nitrosodimethylamine (CCA*is*). Five Syrian hamsters were sacrificed at 1, 2, 3, and 6 months post-infection; photographs were taken for comparison of the gross anatomy of the livers. The protocols were approved by the Animal Ethics Committee of Khon Kaen University (AEKKU43/2553).

Parasite Preparation and Animal Infection

Naturally infected freshwater cyprinoid fish were captured from a water reservoir in an endemic area of opisthorchiasis in Khon Kaen province, northeast Thailand. Fish were minced and digested with pepsin-HCl, then incubated in a shaking water bath at 37 °C for 1 h. The digested fishes were filtered through sieves (1,000, 425 and 106 μm, respectively) and sedimented with 0.85 % NaCl in a sedimentation jar until the supernatant was clear. *O. viverrini* metacercariae were isolated and identified under a stereomicroscope. The characteristic excretory bladder appeared as an oval shape containing a dense mass of dark granules; brownish-yellow pigments were scattered throughout the body. Each Syrian hamster was infected with 50 *O. viverrini* metacercariae via intragastric intubation [11–13].

Prednisolone Preparation

For suppression of host immunity, 5 mg tablets of prednisolone (INPAC Pharma, Thailand) were dissolved with absolute ethanol and then mixed into drinking water [14]. This was orally administered to the assigned groups every day for 2 months (5 mg/kg/d).

Preparation of *N*-nitrosodimethylamine

Diluted *N*-nitrosodimethylamine (NDMA), 12.5 ppm in drinking water, was administered to hamsters beginning at 7 days post-infection and every day thereafter for 2 months in order to induce cholangiocarcinogenesis [13, 15].

Histopathological Observation

Livers and gallbladders from Syrian hamsters in each group were fixed with 10 % formalin and processed in a conventional manner, then stained with hematoxylin and eosin (H&E). Specimens were examined under a light microscope.

Immunohistochemistry for COX2

To determine whether prednisolone had an effect on suppressing inflammation in Syrian hamsters infected with *O. viverrini*, deparaffinized liver tissue sections of OV, OV*is*, CCA and CCA*is* groups at 2 months were processed for immunostaining. Briefly, antigens were retrieved from tissue slides, and the endogenous peroxidase was destroyed. Nonspecific background was blocked with 3 % normal horse serum. Specimens were then incubated with mouse monoclonal antibodies for COX2 (Novocastra Laboratories, Newcastle-upon-Tyne, UK) at a dilution of 1:300 for 1 h at 37 °C, and afterward were washed three times with phosphate buffered saline (PBS). Rabbit anti-goat IgG-HRP (Zymed Laboratories, South San Francisco, CA, USA), diluted 1:300, was used for second antibody labeling. Specimens were incubated with system-HRP labeled polymer anti-rabbit IgG (DacoCytomation, Glostrup, Denmark), and then developed for color with diaminobenzidine as a visual marker. All procedures were performed according to the manufacturer's instructions. A negative control study was performed using normal serum instead of the primary antibody.

Results

Gross Liver Pathology of *O. viverrini* Infection-Associated Cholangiocarcinoma in Immunosuppressed Syrian Hamsters

Gross pathological observation of the livers of uninfected controls (Fig. 1a, f, k, p) and infected groups (Fig. 1b, g, l, q), observed at 1, 2, 3 and 6 month(s), were quite similar and agreed with our previous reports [13, 15]. In brief, the common bile duct and gallbladder in *O. viverrini*-infected groups were slightly enlarged and opaque, with wall thickening in accordance with the duration of infection, and exhibiting a smooth red surface (Fig. 1b, g, l, q). The gross pathological changes in the livers of the *O. viverrini*-infected and immunosuppressed groups (Fig. 1c, h, m, r) were not significantly different.

Syrian hamsters infected with *O. viverrini* and administered *N*-nitrosodimethylamine (cholangiocarcinogenesis models) showed opaque common hepatic bile ducts with rough surfaces (Fig. 1d, i, n, s). Nodularities on liver surfaces and thickening of the common hepatic bile duct walls were observed beginning at 2 months (Fig. 1i), and were

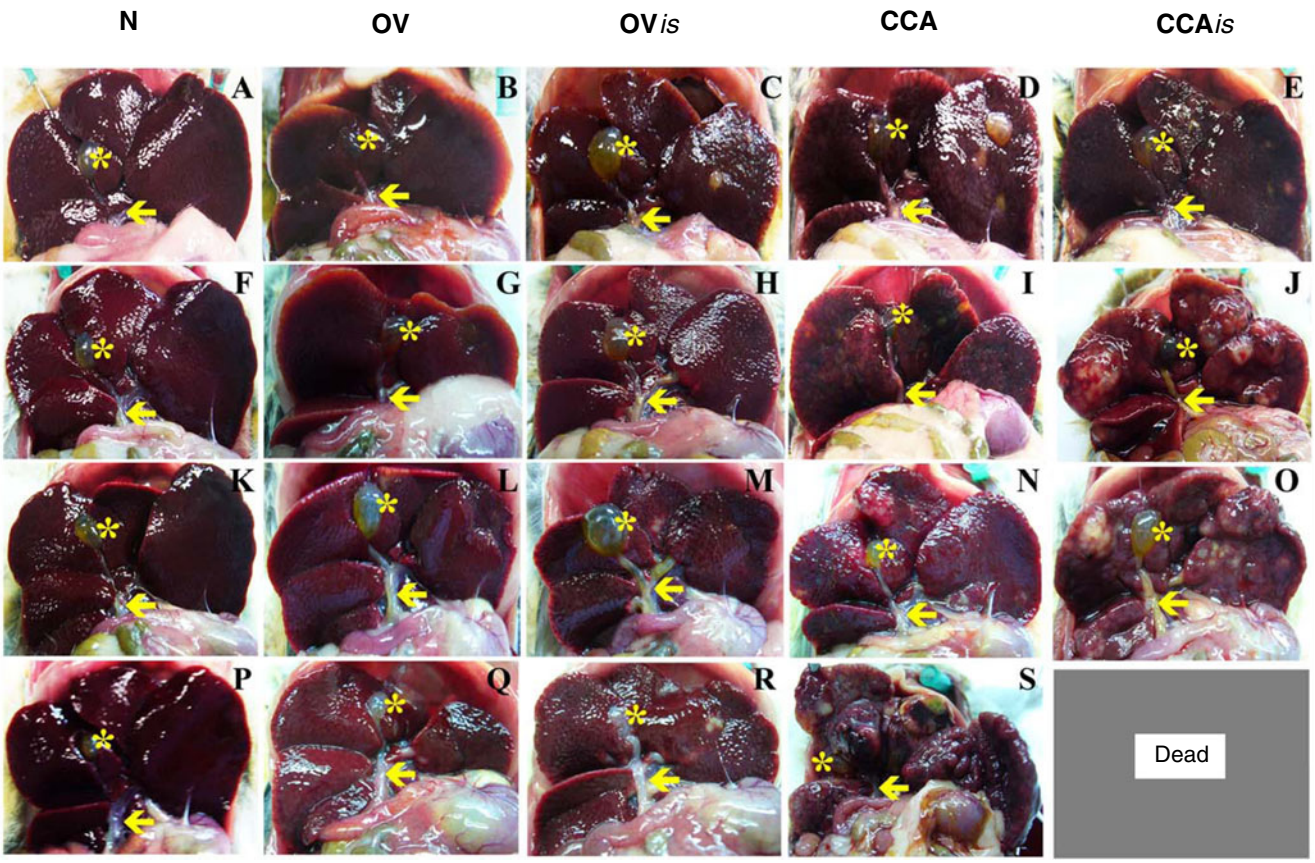

Fig. 1 Representative pictures of livers gross pathology of the Syrian hamster in each group at 1, 2, 3 and 6 month (s) post infection. *N* uninfected group (**a**, **f**, **k**, **p**), OV; *O. viverrini* infected group (**b**, **g**, **l**, **q**), OV*is*; *O. viverrini* infected in immunosuppressive group (**c**, **h**, **m**, **r**), CCA; cholangiocarcinoma group (**d**, **i**, **n**, **s**), CCA*is*; cholangiocarcinoma in immunosuppressive group (**e**, **j**, **o**), * ; gall bladder, *arrow*; common hepatic bile duct

clearly evident at 6 months (Fig. 1s). Interestingly, liver surface nodules and thickening of the common hepatic bile ducts were observed at 2 months in the immunosuppressed cholangiocarcinoma group (Fig. 1j), and were more severe at 3 months (Fig. 1o).

Liver Histopathology of *O. viverrini* Infection-Associated Cholangiocarcinoma in Immunosuppressed Syrian Hamsters

The uninfected control group showed normal liver pathology (Fig. 2a, f, k, p). Surrounding intrahepatic bile ducts and hepatocytes also appeared normal, with no aggregation of inflammatory cells at all times of observation. The histopathological changes in the *O. viverrini*-infected group (Fig. 2b, g, l, q) were similar to the findings in a previous report: aggregation of inflammatory cells surrounding the intrahepatic bile ducts tended to increase at 1 month post-infection, and gradually decreased until 6 months. As expected, in the *O. viverrini*-infected and immunosuppressed group only a few or no inflammatory cells surrounding the intrahepatic bile ducts were found (Fig. 2c, h, m, r). Histopathological changes in the CCA group (Fig. 2d, i, n, s) included many inflammatory cells surrounding the hepatic bile ducts at all times of observation. In addition, at 3 months post-infection in the CCA group (Fig. 2n), an initial cholangiocarcinoma mass was observed, with greater severity at 6 months post-infection (Fig. 2s). These typical histopathological characteristics were correlated with the gross appearances in Fig. 1. Moreover, the histopathologies of the CCA and immunosuppressed groups (Fig. 2e, j, o) were also consistent with the gross pathologies. Only at 2 months post-infection (Fig. 2j) was a cholangiocarcinoma cell mass observed, with early severity at 3 months post-infection, including abundant aggregation of inflammatory cells (Fig. 2o).

COX2 Expression in Cholangiocarcinogenesis of Immunosuppressed Syrian Hamsters

Figure 3 shows the results of immunohistochemical staining for COX2. Generally, COX2 is a key enzyme involved in the process of inflammation and increased expression response to cell stressors such as pro-inflammatory cytokines, growth factors and hormones [16, 17], as well as cholangiocarcinoma cells [18, 19]. In the *O. viverrini*-infected group (Fig. 3a), COX2 was dominantly expressed in the intrahepatic bile duct epithelium. In contrast, the brown color of COX2 staining was not often found in the *O. viverrini*-infected and immunosuppressed group (Fig. 3b). Finally, a comparison of the CCA group with the CCA/immunosuppressed group is demonstrated in Fig. 3c and d, respectively. In the CCA group, many inflammatory cells expressed

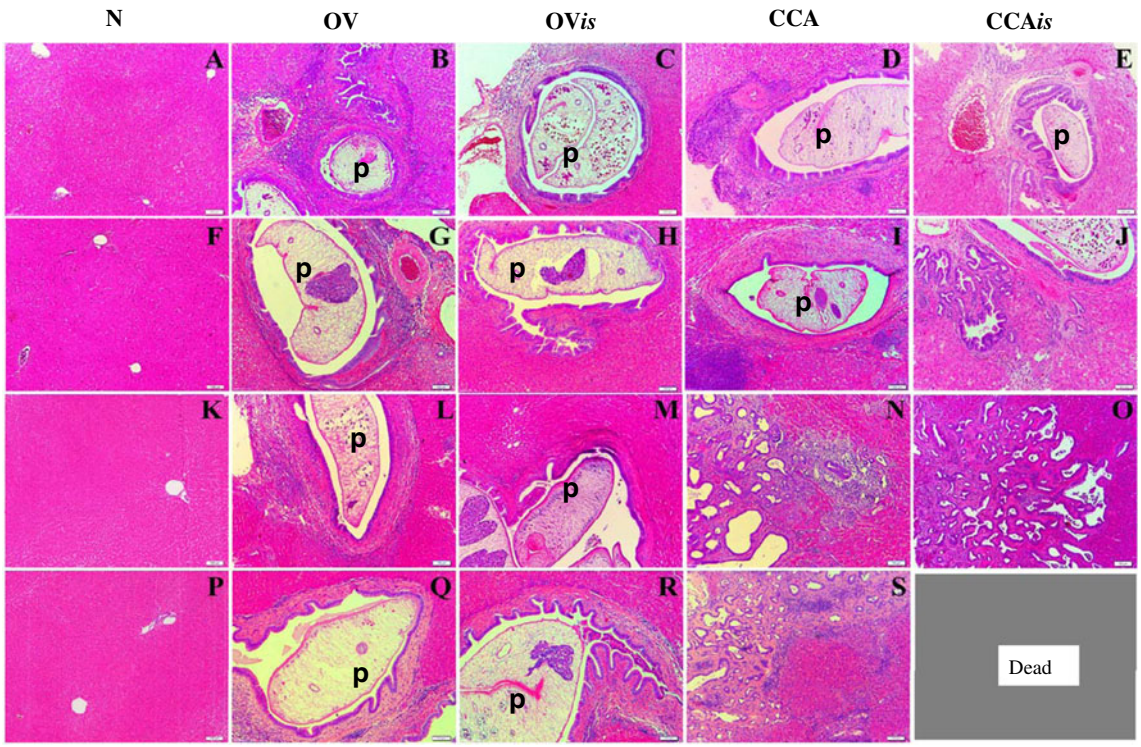

Fig. 2 Representative pictures of liver pathology of the Syrian hamster in each group at 1, 2, 3 and 6 month (s) post infection. N; uninfected group (**a**, **f**, **k**, **p**), OV; *O. viverrini* infected group (**b**, **g**, **l**, **q**), OV*is*; *O. viverrini* infected in immunosuppressive group (**c**, **h**, **m**, **r**), CCA; cholangiocarcinoma group (**d**, **i**, **n**, **s**), CCA*is*; cholangiocarcinoma in immunosuppressive group (**e**, **j**, **o**), p; parasite

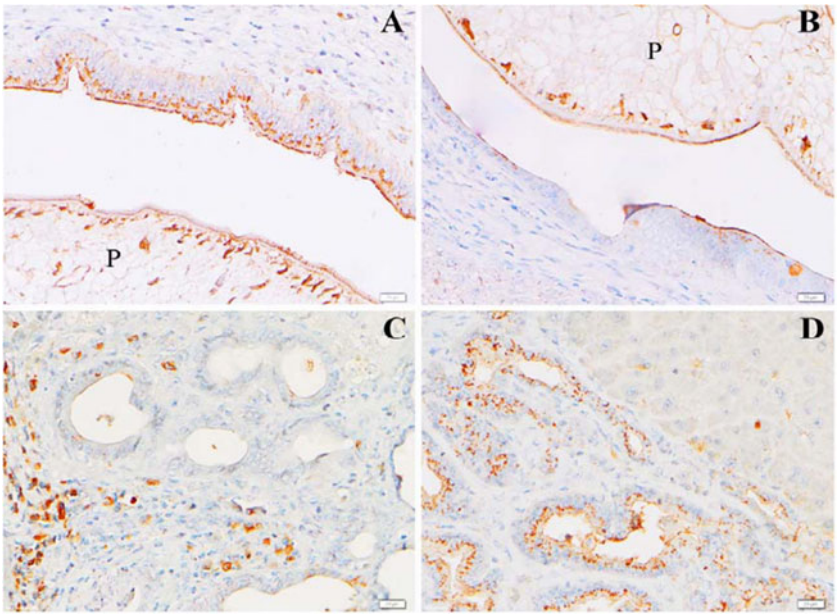

Fig. 3 The immunohistochemistry for COX2 of the Syrian hamster at 2 months post infection (20X magnification). *O. viverrini* infected group (**a**), *O. viverrini* infected in immunosuppressive group (**b**), cholangiocarcinoma group (**c**), cholangiocarcinoma in immunosuppressive group (**d**), P; parasite, brown color; positive staining

COX2, but there was only slight expression in the new bile duct where the cancer mass develops. As anticipated, cholangiocarcinoma in the CCA/immunosuppressed group showed strong expression of COX2 (Fig. 3d).

Discussion

The present study is the first to confirm that immunosuppression by prednisolone induced early cholangiocarcinogenesis in *O. viverrini*-infected Syrian hamsters. Our results showed that although prednisolone had an anti-inflammatory effect by reducing the inflammatory cells surrounding the intrahepatic bile duct in *O. viverrini* infection and NDMA administration groups, this effect was not observed in the CCA model group. Moreover, gross anatomical observation showed an early appearance of CCA in the CCA model group; this finding correlated with the histological result and COX2 immunostaining. These results suggest that immunosuppression with prednisolone enhances early cholangiocarcinogenesis induced by *O. viverrini* infection and NDMA administration.

Generally, host immunity enables the body to protect itself when infected with pathogens. The host will respond by eliminating the pathogens, maintaining a normal condition through both innate and adaptive immunities. In contrast, unbalancing the host immune response can cause many diseases, including cancers [20–23]. It well established that the process of chronic inflammation [24] is a key factor in carcinogenesis by causing DNA damage and mutation. Many cancers are linked to chronic infection or inflammation, such as hepatocellular carcinoma induced by hepatitis B and C [25, 26], and chronic infection with the bacterium *Helicobacter pylori*, which plays a central role in the development of most gastric cancers [21, 22]. Chronic *O. viverrini* infection is a primary cause of the high rate of CCA in the northern part of Thailand; this parasite has been reported to be one of the risks for cholangiocarcinogenesis by the International Agency for Research on Cancer [2].

Prednisolone is one of the glucocorticoid drugs, which can inhibit the expression of many of the genes involved in inflammatory and immune responses that are responsible for inflammation reduction and immune activation in asthma, as well as many other allergic and rheumatoid diseases, inflammatory bowel disease, and systemic diseases including primary sclerosing cholangitis [27–29] which is one of the risk factors for cholangiocarcinoma [30]. In this study, Syrian hamster immunity was suppressed by prednisolone in order to investigate the pathogenic response of the inflammation process in opisthorchiasis-induced cholangiocarcinoma. In the *O. viverrini*-infected and immunosuppressed group, inflammatory cells surrounding the hepatic bile duct were suppressed, as shown in the histopathology results (Fig. 2). Inflammatory responses were investigated by COX2 immunostaining, because several reports have shown that COX2 is involved in the chronic inflammatory process and tumorigenesis. Thus, COX2 inhibitors have been used in many anti-cancer treatments [31, 32]. The study results indicated that inflammatory cells were restricted in the *O. viverrini*-infected and immunosuppressed group, where a reduction of COX2 production was also observed. This may be an effect of the immunosuppressive dose of prednisolone; suppression of inflammatory cells, particularly macrophages, in response to glucocorticoids has been shown to induce macrophage migration inhibitory factor (MIF) [33, 34]. In this case, we can suggest that a decrease of the inflammatory process by prednisolone administration leads to improved hepatic bile duct pathology. This is in agreement with

several reports where glucocorticoids were used for their anti-inflammatory action in the treatment of cancers such as acute and chronic lymphocytic leukemias, Hodgkin's and non-Hodgkin's lymphomas, multiple myeloma, breast cancer, and others [35]. Moreover, prednisolone has been used to reveal complete resolution of the biliary strictures [36], primary sclerosing cholangitis [37] and blood vessel enlargement, sprouting angiogenesis, and lymphangiogenesis [38].

Conversely, we observed early and greater severity of cholangiocarcinogenesis in the CCA/immunosuppressed group; in this group, Syrian hamsters had a high mortality rate and evidence of pathology at 2 months post-infection, earlier in than the normal immunity group. Correlated with the histopathology of this group was observation of early proliferation of hepatic bile duct formation and simultaneous development of cholangiocarcinoma. Moreover, immunohistochemical staining for COX2 was used to determine whether prednisolone inhibits COX2 expression in the processes of cholangiocarcinoma and inflammatory cells, especially macrophages. COX2 was strongly expressed in cholangiocarcinoma cells and macrophages, a finding supported by previous reports on various cancers: colorectal cancer [39–41]; murine skin neoplasms, such as benign papillomas and well-differentiated murine squamous cell carcinomas [42, 43]; bile duct hyperplasia, an early stage of CCA [44]; early-stage human CCA [19]; and the interactions of nitrosamines, *O. viverrini* infection and chronic inflammation in cholangiocarcinogenesis [45]. We suggest that the use of prednisolone for immune suppression may enhance the production of IL-10, an immunoregulatory cytokine that can contribute to suppression of antitumor immunity, and so induced early cancer development in this group. In addition, IL-10 secreted by regulatory T cells (Treg) in response to glucocorticoids (including an increased number of Treg in cancer patients) induces suppression of anticancer immunity and thereby facilitates cancer growth [46–48]. Based on these results, we suggest that when host immunity is suppressed and followed by pathogen-induced carcinogenesis, it may increase inflammatory processes and enhance early carcinogenesis.

Finally, this study confirmed that inflammation is a key component of the cancer microenvironment, and that chronic inflammation plays an important role in carcinogenesis. Moreover, host immune responses are crucially associated with pathogenesis. We can conclude that host immunity is strongly related to pathogenesis in CCA associated with *O. viverrini* infection. Pathology of the hepatic bile duct in cases of *O. viverrini* infection showed improvement when host immune responses were restricted; conversely, cholangiocarcinoma development was enhanced by suppression of host immunity during *O. viverrini* infection. Cholangiocarcinoma development in an immunosuppressed Syrian hamster model implies that cancer risk patients should avoid immunosuppressive therapy, and that balancing of the immune response should be a primary concern in treatment of inflammation-related cancer.

Acknowledgements This research was supported by a grant under the program Strategic Scholarships for Frontier Research Network for the Ph.D. Program Thai Doctoral degree from the Office of the Higher Education Commission, Thailand. The authors also gratefully acknowledge financial support from a Grant-in-Aid for Scientific Research (24590504) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and from the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, through the Health Cluster (SHep-GMS), Khon Kaen University. We also wish to thank the Department of Parasitology, Liver Fluke and Cholangiocarcinoma Research Center, Animal Experimental Unit, Research Affairs, Faculty of Medicine, Khon Kaen University, for giving the research assistant (AS54301) and their assistance.

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