Parasitic diseases remain an unarguable public health problem worldwide. Liver fluke *Clonorchis sinensis* is a high risk pathogenic parasitic helminth which is endemic predominantly in Asian countries, including Korea, China, Taiwan, Vietnam, and the far eastern parts of Russia, and is still actively transmitted. According to the earlier 8th National Survey on the Prevalence of Intestinal Parasitic Infections in 2012, *C. sinensis* was revealed as the parasite with highest prevalence of 1.86% in general population among all parasite species surveyed in Korea. This fluke is now classified under one of the definite Group 1 human biological agents (carcinogens) by International Agency of Research on Cancer (IARC) along with two other parasites, *Opisthorchis viverrini* and *Schistosoma haematobium*. *C. sinensis* infestation is mainly linked to liver and biliary disorders, especially cholangiocarcinoma (CCA). For the purposes of this mini-review, we will only focus on *C. sinensis* and review pathogenesis and carcinogenesis of clonorchiasis, disease condition by *C. sinensis* infestation, and association between *C. sinensis* infestation and CCA. In this presentation, we briefly consider the current scientific status for progression of CCA by heavy *C. sinensis* infestation from the food-borne trematode and development of CCA. [BMB Rep 2016; 49(11): 590-597]

**INTRODUCTION**

Cholangiocarcinoma (CCA) with features of cholangiocytic differentiation is one of the main histological types of malignant tumors of biliary tract epithelia and is a relatively rare type of liver cancer (1). The only therapy for CCA is surgical operation or liver transplantation. Usually, CCA is diagnosed at advanced stages and is considered as an incurable and lethal cancer with poor survival rate of < 24 months (2). This intimidatory cancer develops in the epithelial cells which line the bile ducts and occur in the bile ducts within the liver (intrahepatic), the bile ducts just outside the liver (peripheral) and distal bile ducts. However, this rare tumor is exceptional in regions within Asia, including northeastern Thailand and many areas of southeastern Asia, where infestation with two liver flukes, *Opisthorchis viverrini* and *Clonorchis sinensis* is widespread, respectively (3-5). Due to higher prevalence of liver flukes (a common parasitic infestation) in these areas, there is a higher incidence of CCA (6-8). Therefore, infestations with the two liver flukes are now classified under definite Group 1 biological agents (carcinogens) by the International Agency of Research on Cancer (IARC) based on sufficient evidences in humans (3, 9). Nowadays, three helminth infestations by two food-borne liver flukes, *O. viverrini* and *C. sinensis* and *Schistosoma haematobium* associated with urinary bladder cancer, have been classified under definite group 1 carcinogens. Disease conditions caused by *O. viverrini* and *C. sinensis* infestations are referred to as opisthorchiasis and clonorchiasis, respectively. Although opisthorchiasis and clonorchiasis are the well-known main risk factors of CCA, chronic infection with hepatitis B and C viruses, liver cirrhosis, chronic non-alcoholic liver disease, obesity and hepatolithiasis (gallstones) are also the other minor known risk factors (10). In fact, the connection between CCA and these liver flukes has been the subject of clinical attention for more than 60 years (11, 12). As experimental and epidemiological evidences accumulated, *C. sinensis* infestation strongly implicated the detrimental etiology of CCA with pooled odds ratios between 4.5 and 6.1 (6, 10, 13-15). In this mini-review, we will limit our focus on the association between *C. sinensis* infestation and CCA by briefly summarizing the recent significant scientific progresses (for comprehensive review on *O. viverrini* please refer to work by Sripa B et al.).

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**Keywords:** Cholangiocarcinoma, Clonorchiasis, *Clonorchis sinensis*, Liver cancer, Liver flukes

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LIFE CYCLE OF C. sinensis, SYMPTOMS, DIAGNOSIS AND EPIDEMIOLOGY

C. sinensis is a leaf-shaped slender digenetic trematode, measuring 15-20 mm in length and 3-4 mm in width belonging to class Trematoda, phylum Platyhelminthes. This oriental or Chinese liver fluke is the most pivotal species of food-borne zoonotic parasite in East Asia including Korea, China (except for northwestern regions), Taiwan, northern Vietnam and the far eastern part of Russia, where it is still actively transmitted (17). The life cycle of C. sinensis is characterized by an alternation of sexual and asexual reproductions in three different hosts, such as snails, fish and mammals (18, 19). Embryonated eggs laid by hermaphroditic adult worms are discharged in the biliary ducts and stool of a definite human host. The discharged eggs ingested by a suitable snail intermediate host release miracidia, which go through some developmental stages, such as sporocysts, rediae and cercariae in a regular sequence. The finally developed cercariae in the infected snail are shed into water. These larval stages in the snails reproduce asexually and this reproduction allows for an exponential multiplication of cercariae from a miracidium. After a short period of free-swimming time in water, the shed cercariae meet the 2nd intermediate cyprinid fish, invade the mucous skin and become encysted metacercariae in the subcutaneous tissues or muscles. When a definite mammal host including humans, cats, mink, badgers, rats and dogs eats insufficient cooked, salted, pickled, dried or smoked infested fish, metacercariae separates from the flesh through gastric juice digestion and excyst in the duodenum by a combined action of trypsin and cysteine proteases. Subsequently, the excysted flukes migrate to intrahepatic bile duct through the ampulla of Vater, develop into adult flukes and can dwell for up to 30 years. One worm in human host produces approximately 4000 eggs a day by sexual reproduction (20).

Despite several pathological changes, most of the patients with clonorchiasis in a manner similar to most of the human parasitic infestations have asymptomatic or mild non-specific symptoms except for increased frequency of palpable liver, such as asthenia, nausea, indigestion, headache, dizziness, vertigo, abdominal discomfort, diarrhea, or abdominal pain. Asymptomatic or mild non-specific symptoms may be reflected in the host-parasite relationship, which evolves intimately and progresses in a less harmful way to its host. However, based on few case reports, clinical manifestations caused by clonorchiasis have been mainly related to worm burden (20). Typical physical symptoms of C. sinensis infestation are jaundice, hepatomegaly and liver tenderness (19). Heavy and chronic C. sinensis infestation results in various complications in the liver and biliary systems, primarily cholelithiasis, cholangitis and cholecystitis (21). Growth retardation has been reported in children with heavy infestation. In addition, it is now widely acknowledged that C. sinensis infestation may be associated with CCA. Beyond pathogenesis induced by helminth, hygiene hypothesis and considerable investigations demonstrate sudden rise in epidemic in allergic diseases, such as asthma, anaphylaxis, allergic rhinitis and atopic dermatitis in developed countries; furthermore, this phenomenon has been reported to be lower in developing countries that show a high rate of helminth infestation (22-24). As shown in human and experimental animal models, helminthes are potent immune modulators and induce down T-cell responsiveness, which is partially due to modulation of dendritic cells (DCs) and macrophages (Mφ), and dampens allergic Th2 immune responses through CD4+CD25+Foxp3+ T\textsuperscript{reg} cells. The suppression of airway inflammation in murine asthma model through treatment of C. sinensis-derived total protein is characterized by induction of CD4+CD25+Foxp3+ T\textsuperscript{reg} cell development and modulation of DC functions (25), and a specific C. sinensis-derived antigen demonstrates suppressive skin inflammation through effective mast cell inhibition in allergic and inflammatory diseases (26).

It is noteworthy that parasitic helminthes stimulate some regulatory mechanisms associated with suppression of development of allergies in human and animal models and helminthes are candidates for broader therapeutic application through immune modulation by helminthes (27), although no universal mechanism has yet been elucidated.

The standard diagnosis of clonorchiasis is usually established by microscopic examination of the stool for eggs. The formalin-ether sedimentation technique is known to be more reliable than the direct-smear method for detecting the eggs in stool (28). Although some serological ELISA screening methods for adult C. sinensis antigens are currently available for detection of antibodies, they are not reliably used due to their considerable cross-reactivity and low specificity (29, 30). Application of recombinant proteins for excretory-secretory products (ESPs) of C. sinensis has enhanced the specificity for diagnosis of clonorchiasis (31). Various DNA-based techniques have been developed for the specific detection of C. sinensis (32). Recently, clonorchiasis was commonly diagnosed incidentally during radiological screening, especially by ultrasonography of the abdomen for other purpose, in view of the fact that symptoms of C. sinensis infestation are non-specific in most of the case (33). Praziquantel is a powerful and effective Clonorchis-cidal drug of choice. Recently, tribendimidine, a derivative of amidantel and a broad-spectrum anthelmintic agent, has been acknowledged as an effective and safe agent (34).

As mentioned above, liver fluke infestations occur in some Asian countries where people eat raw (salted, pickled, dried or smoked) or undercooked fish that are infested with these tiny parasite worms. In humans, these flukes dwell in the bile ducts and can cause bile duct cancer. The parasites closely related to risk of developing bile duct cancer are C. sinensis and O. viverrini. In case of C. sinensis, approximately 700 million people are at the risk of infestation and an estimated...
35 million are infested with \textit{C. sinensis} (35). In Korea, according to the most recent 8th National Survey on the Prevalence of Intestinal Parasitic Infections in 2012, \textit{C. sinensis} was revealed as the parasite with highest prevalence of 1.86% in general population as compared to overall prevalence of 2.42% in 2004 (36, 37). In addition, the known endemic regions for \textit{C. sinensis}, especially southern areas along Nakdong and Seomjin rivers, showed high incidence rates of CCA (10, 15, 38). According to 2012 survey data, 0.93 million people were estimated to be infested with clonorchiasis on Korea. However, \textit{C. sinensis} infestation causes one fourth of CCA cases in the endemic area, approximately 10% of CCA cases are estimated to be due to infestation with \textit{C. sinensis}, and estimated CCA relative risk has been continuously rising particularly in areas hyper-endemic for \textit{C. sinensis} infestation. In China, where food-borne parasitic infestations are on a rapid rise, \textit{C. sinensis} infestations have been reported in 27 of 34 provinces and currently the national average prevalence has increased by 75% when compared to the results from the first national survey in 2003 (12.49 million people estimated to be infested with \textit{C. sinensis} with 0.58% prevalence) (39). From a comparative point of view, an enhanced vulnerability to be infested with \textit{C. sinensis} has increased by 75% when compared to the results from the first national survey in 2003 (12.49 million people estimated to be infested with \textit{C. sinensis} with 0.58% prevalence) (39). Although the molecular mechanism involved in the development of CCA are poorly elucidated in detail, it might be simply proposed as a multistep process: normal cholangiocytes $\rightarrow$ pathogen recognition $\rightarrow$ chronic inflammation $\rightarrow$ cell damage $\rightarrow$ reactive cell proliferation $\rightarrow$ genetic/epigenetic mutations $\rightarrow$ malignant cholangiocytes in regular sequence (45). Until date, \textit{C. sinensis}-induced CCA is widely acceptable to be closely linked to chronic inflammation and oxidative stress pathways for creating feasible microenvironment conducive for initiation and promotion of CCA, involving a complex process of several separate mechanisms (4, 46, 47). For pathogen recognition, Toll-like receptors (TLRs) encompass distinctive capacity to sense the initial infection and are the most potent initiators of the inflammatory responses (48). However, prolonged inflammation through excessive production of inflammatory cytokines and chemokines via TLR-mediated signaling could be detrimental because it may cause host toxicity and tissue damage. In the mouse model of clonorchiasis, the expressions of TLR2 and TLR4 were proposed as a multistep process: normal cholangiocytes $\rightarrow$ pathogen recognition $\rightarrow$ chronic inflammation $\rightarrow$ cell damage $\rightarrow$ reactive cell proliferation $\rightarrow$ genetic/epigenetic mutations $\rightarrow$ malignant cholangiocytes in regular sequence (45). Until date, \textit{C. sinensis}-induced CCA is widely acceptable to be closely linked to chronic inflammation and oxidative stress pathways for creating feasible microenvironment conducive for initiation and promotion of CCA, involving a complex process of several separate mechanisms (4, 46, 47). For pathogen recognition, Toll-like receptors (TLRs) encompass distinctive capacity to sense the initial infection and are the most potent initiators of the inflammatory responses (48). However, prolonged inflammation through excessive production of inflammatory cytokines and chemokines via TLR-mediated signaling could be detrimental because it may cause host toxicity and tissue damage. In the mouse model of clonorchiasis, the expressions of TLR2 and TLR4 were upregulated during the infestation of \textit{C. sinensis}, indicating probable participation of TLR2 and TLR4 in the stimulation of innate immune response during \textit{C. sinensis} infestation (49). The T$_{H}$1-based inflammatory consequences instructed by TLRs are not only involved in eliminating pathogenic infections but can also induce fatal pathogenic consequences (50). Similarly, the T$_{H}$2-based pathogen-modulated TLR-mediated signaling event leads to development of immune response beneficial for the pathogen i.e. disease progression. During the chronic \textit{C. sinensis} infestation, clonorchiasis is associated with predominant T$_{H}$2 cytokine production as well as suppression of T$_{H}$1 cytokine production (51, 52). Substantial evidences support the concept that chronic inflammation as a key feature of helminth infestation is linked to various processes involved in carcinogenesis, including cellular transformation, promotion, survival, proliferation, etc (14). In general, inflammation of the bile duct walls is only inconsiderable in regular cases. Sucking onto the biliary epithelium by the fluke results in mechanical tissue damage even at early stages of infestation and, as the fluke matures; the lesion becomes more pronounced and starts to ulcerate (53). Metaplasia of the biliary epithelial cells into

**PATHOGENESIS AND CARCINOGENESIS**

\textit{C. sinensis} causes mechanical injury and inflammation at the environs of biliary tree due to fluke activities, metaplasia of mucin-producing cells in the mucosa, progressive periductal fibrosis and hyperplasia of epithelial cells (40, Fig. 1). The severity of these changes exhibits a tendency to correlate with the duration of fluke infestation, the worm burden, and the susceptibility of the host (41). The pathological changes and the adult flukes might be conducive as a nidus for bacterial infection and intrahepatic stone formation. In addition, the liver flukes secrete or excrete some metabolic products (so-called ESPs), which are highly immunogenic and may be toxic to or interact with the biliary epithelia to stimulate inflammation, promote proliferation and suppress apoptosis (42, 43). Thus, the histopathological changes originate from a combination of mechanical irritation caused by physical contact with infested worms and chemical irritation by their ESPs. Recently analyzed gene expression profiles of three developmental stages of \textit{C. sinensis} might reflect the pathogenesis and carcinogenesis provoked by this liver fluke infestation (44).
mucin-producing cells occurs during very early *C. sinensis* infestation. The mucin-producing cells may proliferate to produce ESPs in the mucosa, leading to persistent and excessive mucus content in the bile (54). This event is initiated by several factors, such as mechanical obstruction of the bile ducts, mechanical injury from the physical activities of feeding and migrating worms, infestation-related inflammation including secondary infection, especially *Escherichia coli*, and toxic effects of ESPs (42, 43, 55-59). Several reports demonstrated that ESPs from adult *C. sinensis* may lead the hyperplasia of normal biliary cells to adenomatous cells with subsequent transformation into CCA by alteration of the transcriptional modification of carcinogenic target genes, such as *Mcm7*, through histone modifications (16, 57). However, the exact mechanism by which carcinogenesis occur remains to be elusive; it is hypothesized that many processes could be implicated. The following possible mechanisms of cholangiocarcinogenesis due to *C. sinensis* infestations have been postulated (16, 61): First, chronic irritation and chronic inflammation caused by the infested *C. sinensis* results in pathologic hyperplasia as a sign of abnormal or precancerous changes and adenomatous changes of bile duct epithelia. The pathologically hyperplastic cells induced by host-parasite interactions due to worm's physical activities are fragile to carcinogen because the biological agent could easily induce DNA damage during active cell proliferation. Second, endogenous oxidative and nitrate DNA damage caused by *C. sinensis* infestation has been studied in both humans and animals (54, 62, 63). It is probable that oxidative lesion products, such as 8-nitroguanine and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OxodG), accumulate in chronic inflammation site around the bile ducts via local nitric oxide production induced by nitric oxide synthase (iNOS) (Fig. 2). Therefore, bile duct epithelial cells are exposed continuously to high concentrations of oxidative lesion contributing to CCA initiation and/or promotion (52, 64). Third, *C. sinensis*-induced redox imbalance is due to the enzymatic trigger for production of drug metabolizing enzymes and free-radical generating enzymes (65): For example, experimentally cytochrome P-450 in *C. sinensis* is responsible for metabolism in the worms and for detoxification contributed to worm survival and drug resistance. Also, free radicals generated by *C. sinensis* infestation play a critical role in triggering NF-κB-mediated inflammation (57). ESPs of *C. sinensis* can induce recruitment of histone acetyltransferases (HAT) and regulation of minichromosome maintenance (Mcm) proteins for the physiological hyperplasia (57). Fourth, recent evidences have shown modulation of carcinogenesis prevention processes as one of the multiple cholangiocarcinogenic pathways, for example, involvement of small non-protein-coding RNAs (microRNA). Indeed, it is now generally accepted that microRNAs serve as a negative gene regulator by participating in the modulation of a variety of physiological pathways and have the potential to control various gene targets (66). Recent finding indicates that, during *C. sinensis*-associated cholangiocarcinogenesis in animal model and humans, microRNAs function as both tumor suppressors and oncogenes (67). In addition, IL-6 over-expressing malignant cholangiocytes have been reported to modulate the expression of DNA methyltransferase 1 in a microRNA-dependent manner (68). In the case of carcinogenetic pathway induced by *C. sinensis* infestation, exposure of human HuCCT1 CCA cells to ESPs for different periods of time as compared to normal H69 cholangiocytes has shown differentially altered microRNA profile changes revealing the involvement of microRNA in cell proliferation, inflammation, oncogene activation/suppression, migration/invasion/metastasis, and DNA methylation (59).

Inflammation drives generation of free radicals (reactive oxygen species (ROS) and reactive nitrogen species (RNS)), which leads to lipid peroxidation (LPO), and promotes the acquisition of considerable oxidative DNA damage and dysregulation of cell homeostasis (Fig. 2). Considerable reports have demonstrated that ROS are involved in the link between chronic inflammation and cancer (69, 70). For example, exposure of human HuCCT1 CCA cells to *C. sinensis* ESPs revealed enhanced generation of free radicals by activation of NADPH oxidase (NOX), xanthine oxidase (XO), lipoygenase

![Fig. 2. Possible link of liver fluke *C. sinensis*-induced redox imbalance with CCA development.](image-url)
(LO), cyclooxygenase (COX) and iNOS (59). In the mouse infectious model for \textit{C. sinensis}, liver fluke infestation differentially elevated secretion of proinflammatory cytokines such as TNF-\(\alpha\), IL-1\(\beta\) and IL-6, indicating that, under the chronic inflammation states, persistent and dysregulated expressions of these pleiotropic cytokines are promutagenic for malignant cell transformation (4, 47). Chronic and elevated signaling events by TNF-\(\alpha\) and IL-1\(\beta\) transactivation of NF-\(\kappa\)B, which in turn induces the proinflammatory mediating genes including iNOS, IL-6, etc, results in amplification of inflammation (71). Moreover, substantial evidences have demonstrated that nitric oxide (NO) is not only cytotoxic but may also be genotoxic leading to DNA damage. The main role of nitric oxide (synthesized by iNOS after challenge by immunological and inflammatory stimuli) during inflammation involves triggering of carcinogenesis through accumulation of DNA damage by inhibiting DNA repair system and stimulation of COX-2 expression (72, 73). Furthermore, LPO products, such as trans-4-hydroxy-2-nonenal (HNE), malondialdehyde (MDA) and crotonaldehyde, can modulate the 2\(^{nd}\) messenger systems involved in inflammation and carcinogenesis for increasing cell proliferation and decreasing apoptosis in the initiated cell population (74, 75). Additional critical connection between chronic inflammation and cancer development is cyclooxygenase (COX-) and lipoxygenase (LOX)-catalyzed arachidonic and linoleic acid metabolism (76, 77). Based on the experiment in \textit{C. sinensis}-infested mouse liver tissues, expressions of COX-2 and 5-LOX with increased 8-OxodG accumulation in the nucleus of the cells with inflammation were intensively detected in the inflammatory nidus (47). COX-2, an inducible form of COX, is stimulated by cytokines and lipopolysaccharide and mainly expressed during the inflammation responsible for stimulating cell growth (78). In case of RNS, N-nitrosodimethylamine (NDMA), one of the products of endogenous nitrosation, is significantly metabolized by cytochrome P-450. At intracellular level, exposure of HEK293T to ESPs of \textit{C. sinensis} with NDMA is responsible for proliferation in the G2/M phase and expression of cell cycle related proteins, such as E2F1, phosphorylated RB and cyclin B (42, 79). In a study on Syrian golden hamster (experimental model), the mechanical and chemical irritation caused by \textit{C. sinensis} worm and NDMA was considered as a probable cause for genetic alterations leading to neoplastic transformation by producing aberrant proteins including a novel oncogene \textit{PSMD10}, cyclin-dependent kinase 4 gene CDK4, tumor suppressor gene \(p53\) and protein retinoblastoma (RB) and leading to enhanced survival of the transformed bile duct cells through BAX and caspase 9 (80). The researchers provided the evidence on coordination between changes in the levels of gene and protein expression profile and histopathological changes in \textit{C. sinensis} and NDMA-induced CCA model.

**CONCLUDING REMARKS**

It is undeniable that DNA damage caused by \textit{C. sinensis} infestation is provoked in biliary epithelia, while proper homeostatic mechanisms are dysregulated, resulting in genetic alterations that might be indigenous to the biliary tract, thus leading to malignant transformation. The implicated mechanisms in promotion of malignancy from a parasite infestation discussed in the present review includes mechanical and chemical irritation, chronic inflammation, genomic instability, transcriptomic, proteomic and microRNA profile alterations by ESPs, and dysregulation of immune response. However, it seems that carcinogenesis associated with \textit{C. sinensis} can be provoked by various mechanisms and may still be a colossal subject to be elucidated. Moreover, low incidence of CCA in some areas showing a high prevalence of \textit{O. viverrini} and \textit{C. sinensis} indicates that other factors are pivotally involved in cholangiocarcinogenesis. Animal studies demonstrate that, in the absence of other carcinogens, CCA is unlikely to develop into liver fluke infestation. Consequently, it is proposed that all the described possible mechanisms could be apprehended in a concert during the development of CCA. So these liver flukes are mainly promoters and not initiators of CCA. It is also necessary that, for the discovery of biomarkers for early diagnosis and discrimination of disease from HBV infection, which is highly prevalent in many clonorchiasis-endemic areas, morbidity due to \textit{C. sinensis} infestation and drivers of carcinogenesis by chronic infestation should be assessed. Furthermore, for control and elimination of clonorchiasis, rapid immunological tools based on the mathematical modeling need to be developed. In conclusion, this brief review provides tiny aspects on current knowledge on the association of \textit{C. sinensis} infestations with CCA formation, and further elucidation in future experimental and clinical based researches is necessitated.

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