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# ***Clonorchis sinensis***, an Oriental Liver Fluke, as a Human Biological Agent (Carcinogen) of Cholangiocarcinoma: A Brief Review.

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**Running Title:** Association between *Clonorchis sinensis* and CCA

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## ABSTRACT

Parasitic diseases remain an unarguable public health problem in the world. Liver fluke *Clonorchis sinensis* is a high risk pathogenic parasitic helminth endemic predominantly in Asian countries, including Korea, China, Taiwan, Vietnam, and the far eastern part of Russia, which still actively transmitted. According to the last 8$^{th}$ National Survey on the Prevalence of Intestinal Parasitic Infections in 2012, *C. sinensis*, whose prevalence was 1.86% in general population, revealed the highest prevalence parasite among all parasite species surveyed in Korea. This fluke is now classified in one of definite Group 1 human biological agents (carcinogens) by International Agency of Research on Cancer (IARC) together 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 to *C. sinensis* and review pathogenesis and carcinogenesis of clonorchiasis, disease condition by *C. sinensis* infestation, and association between *C. sinensis* infestation and CCA. Here, we briefly consider the current scientific status for progression of CCA by heavy *C. sinensis* infestation from this food-borne trematode and development of CCA.

## Introduction

Cholangiocarcinoma (CCA) with features of cholangiocyte 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 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 of the liver (perihilar) and distal bile ducts. However, this rare tumor is except in regions within Asia, including northeastern Thailand and many areas of southeastern Asia, where infestation with two liver flukes, *Opisthorchis viverrini* and *Clonorchis sinensis*, respectively, is widespread (3, 4, 5). Due to a higher prevalence of these liver flukes, a common parasitic infestation, there is a higher incidence of CCA in these areas (6, 7, 8). Therefore, infestations with these two liver flukes, *O. viverrini* and *C. sinensis*, are now both classified in 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 as the definite group 1 carcinogens. Disease conditions by *O. viverrini* and *C. sinensis* infestations are called 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 have been the subject of clinical attentions for more than 60 years (11, 12). As experimental and epidemiological evidences accrued, *C. sinensis* infestation strongly implicates the detrimental etiology of CCA with pooled odds ratios between 4.5 and

6.1 (6, 10, 13, 14, 15). In this mini-review, we will limit our focus about the association between *C. sinensis* infestation and CCA by briefly summarizing the recent significant scientific progresses (for comprehensive review on *O. viverrini* see **ref.** 16).

**Life cycle of *C. sinensis*, symptom, diagnosis and epidemiology**

*C. sinensis* is a leaf-shaped slender digenetic trematode, 15-20 mm long and 3-4 mm wide. This oriental or Chinese liver fluke is the most pivotal species of food-borne zoonotic parasite in the class Trematoda, phylum Platyhelminthes in East Asia including Korea, China (except northwestern regions), Taiwan, northern Vietnam and the far eastern part of Russia, where 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 hermaphroditical 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, radiae and cercariae in 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 2$^{nd}$ 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 separate from the flesh through gastric juice digestion and excyst in the duodenum by a combined action of trypsin and cysteine proteases. Then, the excysted flukes migrate to intrahepatic bile duct through the ampulla of Vater, develop into adult flukes and can dwell them 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 patients with clonorchiasis like most human parasitic infestations have asymptomatic or mild non-specific symptoms except an increased frequency of palpable liver, such as asthenia, nausea, indigestion, headache, dizziness, vertigo, abdominal discomfort, diarrhea, or abdominal pain. It may be reflected in the host-parasite relationship evolved intimately and progressed less harmful to its host. However, from case reports, clinical manifestations caused by clonorchiasis are 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, mainly 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 that epidemic in allergic diseases, such as asthma, anaphylaxis, allergic rhinitis and atopic dermatitis, has been suddenly rising in developed countries and that this phenomenon is definitely lower in developing countries that show a high rate of helminth infestation (22, 23, 24). As shown in humans 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 (M$\phi$), and dampens allergic $T_H2$ immune responses through $CD4^+CD25^+Foxp3^+$ $T_{reg}$ cells. The suppression of airway inflammation in murine asthma model with treatment of *C. sinensis*-derived total protein is characterized by inducing $CD4^+CD25^+Foxp3^+$ $T_{reg}$ cell development and modulating DC functions (25), and a specific *C. sinensis*-derived antigen shows the suppressive skin inflammation through effective mast cell inhibition for allergic and inflammatory diseases (26). It is significant that parasitic helminthes stimulate some

regulatory mechanisms associated with suppression of development of allergies in humans 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* raises specificity for the diagnosis (31). Various DNA-based techniques have been developed for the specific detection of *C. sinensis* (32). Recently, clonorchiasis is commonly diagnosed incidentally during radiological screening, especially by ultrasonography of the abdomen for other purpose, since symptoms of *C. sinensis* infestation are nonspecific in most 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 above mentioned, liver fluke infestations occur in some Asian countries when people eat raw (salted, pickled, dried or smoked) or inappropriately 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 ones most closely related to bile duct cancer risk are *C. sinensis* and *O. viverrini*. In case of *C. sinensis*, approximately 700 million people are to be at risk of infestation and an estimated 35 million are infested with *C. sinensis* (35). In Korea, according to the last 8$^{th}$ National Survey on the Prevalence of Intestinal Parasitic Infections in 2012, the parasite of the highest infestation rate was *C. sinensis*, whose prevalence was 1.86%

in general population compared to overall 2.42% prevalence within the population in 2004 (36, 37). In addition, the known regions of *C. sinensis* endemicity, especially southern areas along Nakdong and Seomjin Rivers, showed high incidence rates of CCA (10, 15, 38). The 2012 survey data estimate 0.93 million people for clonorchiasis on Korea. However, *C. sinensis* infestation causes one fourth of CCA cases in the endemic area, approximately 10% of CCA cases are estimated due to infestation with *C. sinensis*, and estimated CCA relative risk has been continuously raising particularly in areas hyper-endemic for *C. sinensis* infestation. In China, as one of the fastest increasing food-borne parasitic infestations, *C. sinensis* infestations have been reported in 27 of 34 provinces and the national average prevalence has increased by 75% compared to the results of the first national survey, with an estimated 12.49 million people (0.58% prevalence) being infestation in 2003 (39). In comparison, an enhanced susceptibility to CCA raising in patients with *O. viverrini* infestation has been reported from Thailand (4).

**Pathogenesis and carcinogenesis**

*C. sinensis* causes mechanical injury and inflammation at the environs of biliary tree from the 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 shows a tendency to correlate with the duration of fluke infestation, the worm burden, and the susceptibility of the host (41). These pathological changes and the adult flukes might be conducive as a nidus for bacterial infection and intrahepatic stone formation. In addition, these 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, these histopathological changes are originated from a combination of mechanical irritation by

physical contact with infested worms and chemical irritation by their ESPs. Recently analyzed gene expression profiles of three developmental stages of *C. sinensis* might reflect the pathogenesis and carcinogenesis provoked by this liver fluke infestation (44).

Although the molecular mechanisms involved in the development of CCA are poorly elucidated in detail, it might be simply proposed as a multistep process: normal cholangiocytes → pathogen recognition → chronic inflammation → cell damage → reactive cell proliferation → genetic/epigenetic mutations → malignant cholangiocytes in regular sequence (45). Up-to-date, *C. sinensis*-induced CCA is widely acceptable to be closely linked to chronic inflammation and oxidative stress pathways for feasible microenvironment conducive to initiation and promotion of CCA, involving a complex process of several separate mechanisms (4, 46, 47). For pathogen recognition, Toll-like receptors (TLRs) have unique capacity to be scrutable 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 expression of TLR2 and TLR4 were upregulated during the infestation of *C. sinensis*, indicating that both TLR2 and TLR4 probably participate in the stimulation of the innate immune response during *C. sinensis* infestation (49). $T_H1$-based inflammatory consequences instructed by TLRs not only involve in eliminating pathogenic infections but also can induce fatal pathogenic results (50). Similarly, the $T_H2$-based pathogen-modulated TLR-mediated signaling event develops immune response beneficial for the pathogen *i.e.* disease progression. During the chronic *C. sinensis* infestation, clonorchiasis is associated with predominant $T_H2$ cytokine production as well as suppression of $T_H1$ cytokine production (51, 52). Substantial evidences support 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 early in its infestation and, as the fluke mature, the lesion is more pronounced and ulcerates (53). Metaplasia of the biliary epithelial cells into mucin-producing cells occurs during very early *C. sinensis* infestation. These 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, 56, 57, 58, 59). Several reports demonstrated that ESPs from adult *C. sinensis* provoke the profile changes in transcriptome, proteome and microRNA expression in human HuCCT1 CCA cells and in mouse liver (56, 58, 60, 61). Moreover, ESPs from *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 occurs remains to be elusive; many processes could be implicated. The following possible mechanisms of cholangiocarcinogenesis due to *C. sinensis* infestations have been postulated (16, 62): 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. These pathologically hyperplastic cells induced by host-parasite interactions due to worm's physical activities are brittle to carcinogen because the biological agent could easily induce DNA damage during active cell proliferation. Second, endogenous oxidative and nitrative DNA damage caused by *C. sinensis* infestation has been studied in both humans and animals (54, 63, 64). It is probably that

oxidative lesion products, such as 8-nitroguanine and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OxodG), accumulates in chronic inflammation site around the bile ducts *via* local nitric oxide by inducible nitric oxide synthase (*i*NOS) (**Fig. 2**). Therefore, bile duct epithelial cells are exposed continuously to high concentrations of oxidative lesion contributing to CCA initiation and/or promotion (52, 65). Third, *C. sinensis*-induced redox imbalance is due to the enzymatic trigger for drug metabolizing enzymes and free-radical generating enzymes (66): For example, experimentally cytochrome P-450 in *C. sinensis* is responsible for the worm metabolism and detoxification contributed to worm survival and drug resistance. Also generated free radicals by *C. sinensis* infestation play a critical role in triggering NF-κB-mediated inflammation (57). ESPs of *C. sinensis* can induce histone acetyltransferases (HAT) recruitment and regulation of minichromosome maintenance (Mcm) proteins for the physiological hyperplasia (57). Fourth, recent evidences have shown the modulation of carcinogenesis prevention processes as one of the multiple cholangiocarcinogenic pathways, for example, involvement of small non-protein-coding RNAs, so-called microRNA. Indeed, it is now generally accepted that microRNAs as a negative gene regulator participating in the modulation of a variety of physiological pathways have the potential to control various gene targets (67). Recent finding indicates that, during *C. sinensis*-associated cholangio-carcinogenesis in animal model and human samples, microRNAs function as both tumor suppressors and oncogenes (68). In addition, IL-6 overexpressing malignant cholangiocytes could modulate the expression of DNA methyltransferase 1 in a microRNA-dependent manner (69). In the case of carcinogenetic pathway by *C. sinensis* infestation, treatment of ESPs into human HuCCT1 CCA cells for different periods of time compared to normal H69 cholangiocyte cells has shown differentially altered microRNA profile changes revealing involvement in cell proliferation, inflammation, oncogene activation/suppression, migration/invasion/metastasis, and DNA methylation (61).

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 (70, 71). For example, introduction of *C. sinensis* ESPs to human HuCCT1 CCA cells showed increases in free radicals generated by the activation of NADPH oxidase (NOX), xanthine oxidase (XO), lipoxygenase (LO), cyclooxygenase (COX) and *i*NOS (61). In the mouse infectious model for *C. sinensis*, liver fluke infestation differentially elevates the secretion of proinflammatory cytokines such as TNF-α, IL-1β and IL-6, indicating that, under the chronic inflammation states, persistent and dysregulated expressions of these pleiotropic cytokines are to be promutagenic for malignant cell transformation (4, 47). Chronic and elevated signaling events by TNF-α and IL-1β □□□□□□□□□□□□ transactivation of NF-κB, which in turn induces the proinflammatory mediating genes including iNOS, IL-6 *etc*, resulting in amplification of inflammation (72). Moreover, substantial evidences demonstrated that nitric oxide (NO) is not only cytotoxic but may also be genotoxic leading to DNA damage. The main part of nitric oxide during inflammation, which triggers the process of carcinogenesis through accumulation of DNA damage by inhibiting DNA repair system and stimulating COX-2 expression (73), is synthesized by *i*NOS after challenge by immunological and inflammatory stimuli (74). As well as free radicals, 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 in cell proliferation and decreasing in apoptosis of the initiated cell population (75, 76). Additional critical connection between chronic inflammation and cancer development is cyclooxygenase(COX)- and lipoxygenase (LOX)-catalyzed arachidonic and linoleic acid metabolism (77, 78). From the experiment in *C. sinensis*-

infested mouse liver tissues, expressions of COX-2 and 5-LOX with increased 8-OxodG accumulation in the nucleus of the inflammation cells are 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 the stimulating cell growth (79). In case of RNS, N-nitrosodimethylamine (NDMA), one of the products of endogenous nitrosation, is significantly metabolized by cytochrome P-450. In intracellular level, ESPs of *C. sinensis* with NDMA to HEK293T are responsible for the proliferation in the G2/M phase and expression of cell cycle related proteins, such as E2F1, phosphorylated RB and cyclin B (42, 80). In Syrian golden hamster as an experimental model, the mechanical and chemical irritation with *C. sinensis* worm and NDMA may cause genetic alterations leading to neoplastic transformation by producing aberrant proteins including a novel oncogene *PSMD10*, cyclin-dependent kinase 4 gene *CDK4*, tumor suppressor gene *p53* and protein retinoblastoma (RB) and making more survival of the transformed bile duct cells through *BAX* and *caspase 9* (81). They provided the evidence that the expression profile changes in the levels of gene and protein is well matched with the histopathological changes in *C. sinensis* and NDMA-induced CCA model.

## Concluding remarks

Indeed, DNA damage caused by *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, leading to malignant transformation. The implicated mechanism of promotion of malignancy from a parasite infestation discussed here includes mechanical and chemical irritation, chronic inflammation, genomic instability, transcriptiomic, proteomic and microRNA profile alterations by ESPs, and dysregulation of immune response. However, it seems that carcinogenesis associated with *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 *O. viverrini* and *C. sinensis* indicates that other factors are pivotally involved in cholangiocarcinogenesis. Animal studies demonstrate that, without other carcinogens, CCA is improbable to develop in liver fluke infestation. Thus all the described possible mechanisms may be concerned in 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 the discriminability of disease from HBV infection highly prevalent in many clonorchiasis-endemic areas, morbidity due to *C. sinensis* infestation and drivers of carcinogenesis by chronic infestation should be assessed and that, for the control and elimination of clonorchiasis, rapid immunological tools based on the mathematical modeling have to be developed. In conclusion, this brief review shed tiny aspect on current knowledge on the association of *C. sinensis* infestations and CCA formation, which needs to be elucidated in future experimental and clinical based researches.

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**Figure legends**

**Fig. 1.** Histopathological liver-section image of clonorchiasis (hematoxylin and eosin staining) at the 4$^{th}$ week of *C. sinensis* post-infestation.

**Fig. 2.** Possible link of liver fluke *C. sinensis*-induced redox imbalance in CCA development.

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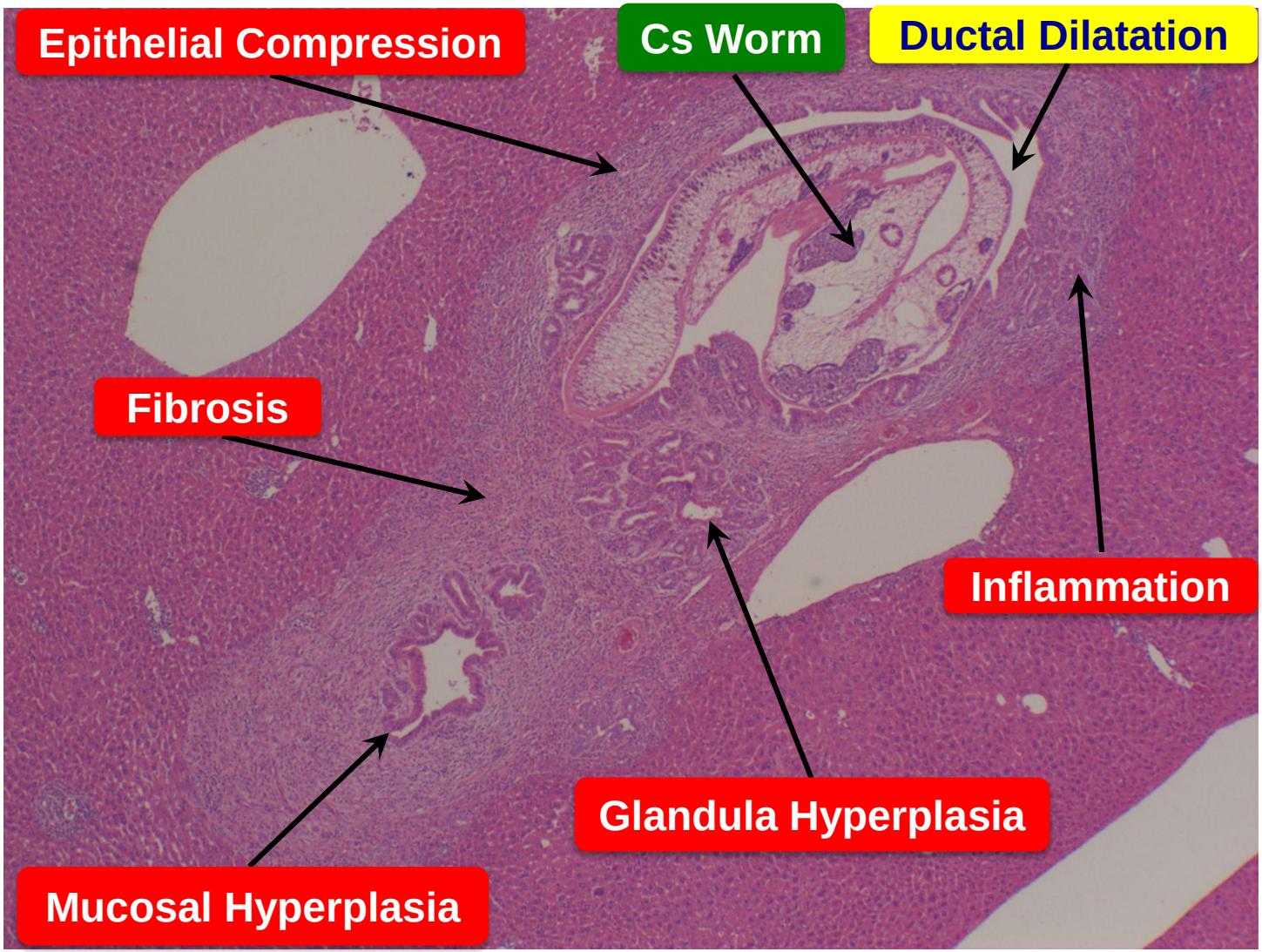


**Fig. 1. Kim *et al.***

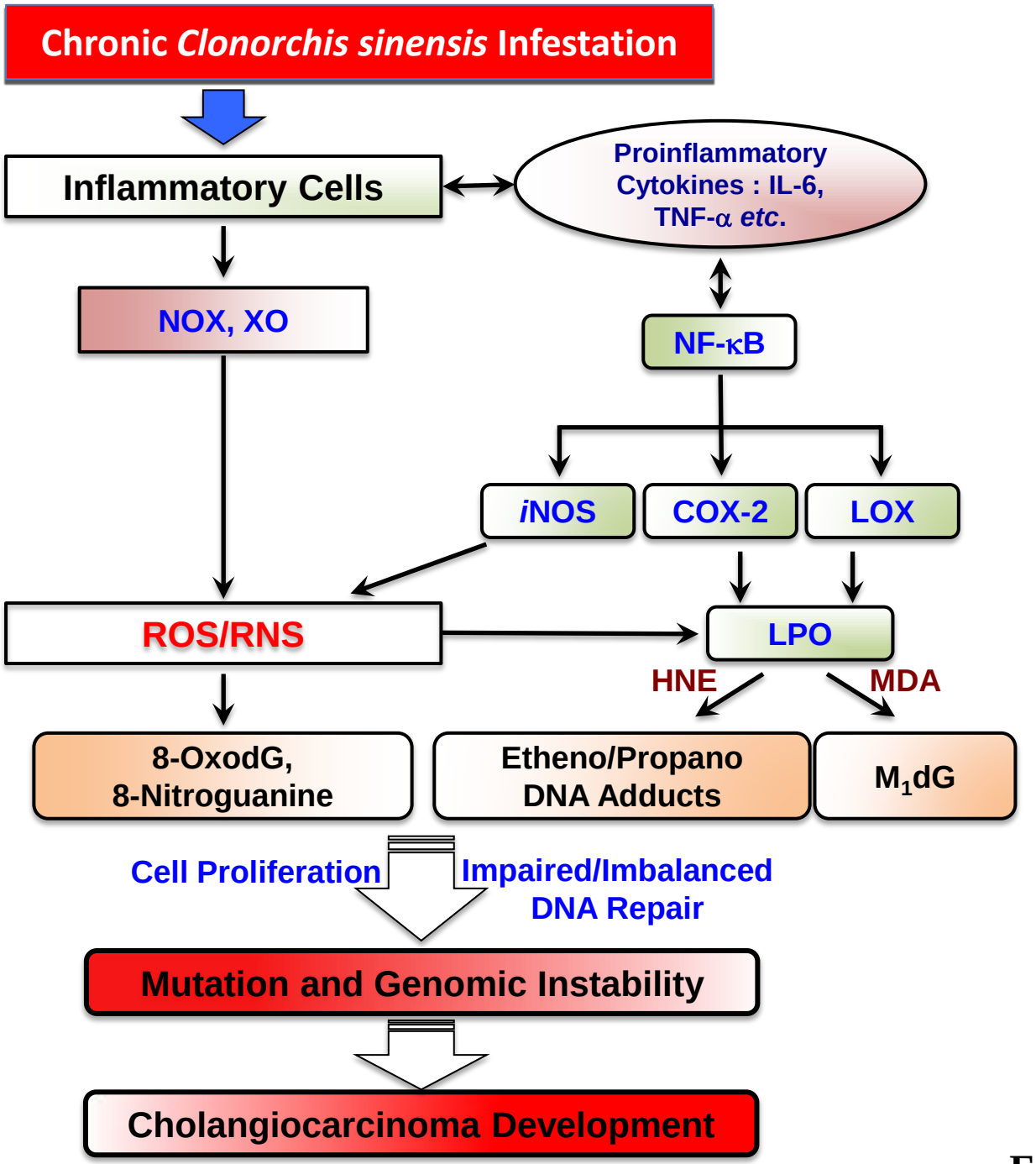


**Fig. 2. Kim *et al.***