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Co-infections with liver fluke and *Helicobacter* species: A paradigm change in pathogenesis of opisthorchiasis and cholangiocarcinoma?

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Abstract

Infection with the fish-borne liver fluke *Opisthorchis viverrini* is classified by the International Agency for Research on Cancer as a Group 1 carcinogen: definitely carcinogenic in humans. Cofactors likely contribute to bile duct cancer (cholangiocarcinoma) caused by this infection. Here we review recent findings that address the role of liver fluke associated *H. pylori* in hepatobiliary disease and malignancy. We hypothesize that co-infection by *O. viverrini* and the bacillus *Helicobacter pylori* is central of liver fluke infection associated cholangiocarcinoma.

Graphical Abstract

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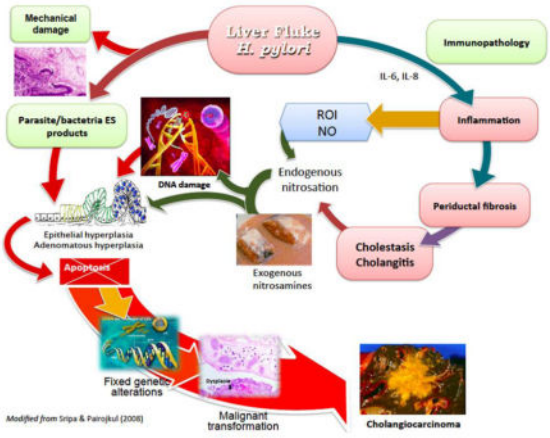

Keywords

Liver fluke; *Opisthorchis viverrini*; *Helicobacter* spp; Co-infection; pathogenesis; hepatobiliary diseases; cholangiocarcinoma

1. Introduction

Foodborne trematodiasis caused by infection with the opisthorchiid liver flukes *Opisthorchis viverrini, O. felineus* and *Clonorchis sinensis* remains a major public health problem in East Asia and Eastern Europe where >40 million people are infected [1, 2]. *O. viverrini* is endemic in Thailand, Lao People's Democratic Republic (Lao PDR), Vietnam and Cambodia with over 10 million people are infected [1]. Humans acquire the infection by eating raw or undercooked fish harboring infective stage metacercariae (reviewed in [3]). Upon ingestion, the metacercariae excyst in the duodenum and juvenile flukes migrate into the biliary tree. In the bile ducts, the parasites mature over four weeks into adult flukes. Parasites eggs are shed in the fecal stream to the environment where the eggs are ingested by freshwater snails of the genus *Bithynia*. The parasite undergoes transformations and multiplications within the snail, culminating in the release of cercariae that seek out and penetrate the skin of a freshwater cyprinid fish, completing the cycle. Human infection causes several hepatobiliary abnormalities, including cholangitis, obstructive jaundice, hepatomegaly, periductal fibrosis, cholecystitis and cholelithiasis (see [3]). Both experimental and epidemiological evidence strongly implicates liver fluke infection in the etiology of one of the primary liver cancer subtypes – cholangiocarcinoma (CCA), a fatal bile duct cancer [1, 4, 5]. Khon Kaen province in north-eastern Thailand where the *O. viverrini* liver fluke is endemic has reported the highest incidence of CCA in the world, >100 cases per 100,000 [6]. However, additional risk factors for hepatobiliary diseases and CCA have been documented including primary sclerosing cholangitis (see [7]), inflammatory bowel disease [8], metabolic syndromes [9], hepatitis virus [10], fluke infection-associated oxysterols [11], and infection with *Helicobacter* spp. [12]. The last is attracting increasing research interest [13].

2. *Helicobacter* spp. and extragastric diseases

Infection with *Helicobacter pylori,* a Gram-negative bacillus is the first bacterial infection known to be an etiological agent of gastric diseases including gastric adenocarcinoma [14–17]. Virulence factors of *H. pylori* including cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA) contribute to the pathogenesis of *H. pylori*-associated disease [18, 19]. Although chronic *H. pylori* infection is associated with the stomach possible association with several extragastric complications including hepatobiliary and pancreatic diseases have been proposed [20, 21]. There is strong evidence that *H. pylori* seropositivity and biliary tract cancer with overall OR 5.47 and, specifically, for extrahepatic (OR 7.01) and intrahepatic cancer (OR 10.67) but not for hepatocellular carcinoma in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) cohort [22]. For liver fibrosis, prevalence of *cag*A *H. pylori* was directly proportional to severity of liver disease and was more positive in advanced stages of fibrosis (28.2%) compared to early stages (5.9%) in HCV-related chronic hepatitis and cirrhosis [23]. The mechanism by which *H. pylori* induces liver fibrosis may involve increased cytokines, i.e. TGF-β1 and oxidative stress induced pro-inflammatory signaling pathways in hepatic stellate cell line (HSC) [24, 25]. Other species of *Helicobacter*, specifically *H. hepaticus* and *H. bilis* also are implicated in hepatobiliary disease [12, 13, 22].

3. Associations among *Helicobacter,* opisthorchiasis and cholangiocarcinoma

H. pylori has been reported to be involved in a case series of hepatobiliary diseases in opisthorchiasis endemic Thailand [26]. We first systematically reported an association between *H. pylori,* specifically *cagA* positive-*H. pylori* and CCA but not hepatolithiasis or normal controls in patients from Northeast Thailand, a region endemic for opisthorchiasis [26, 27]. CCA cases with *H. pylori* infection exhibit higher portal inflammation and biliary cell proliferation as determined by PCNA immunohistochemistry [26]. Molecular mechanisms integral to *H. pylori* induced hepatobiliary diseases have also been described [28–30]. Since CCA is strongly associated with opisthorchiasis in endemic areas, as noted, *O. viverrini* may have an integral though still cryptic relationship with *H. pylori*. Indeed, we have been aware of this relationship for some time, and recently reported an association between *O. viverrini* and *Helicobacter* spp. in a hamster model [31]. The liver fluke infected hamsters showed significantly higher *H. pylori* and *H. bilis* than control, non-liver fluke-infected hamsters. In addition, *H. pylori* can be detected in the gut epithelium of *O. viverrini* and hence we have concluded that the liver fluke represents a reservoir of *H. pylori within the biliary system* [31]. Similar findings have been seen in humans infected with *O. viverrini*. The higher the liver fluke infection intensity, as determined by *O. viverrini* eggs per gram of feces, the greater the fecal numbers of *H. pylori*. Moreover, we also demonstrated that *cagA* positive *H. pylori* associated with increased risk of periductal fibrosis as determined by ultrasonography in opisthorchiasis (Deenonpoe et al., manuscript submitted). Given our pioneering research in the pathogenesis of liver fluke induced pathology and CCA and over 20 years research experience in this field [2, 3, 11, 29, 32, 33], we hypothesize that the liver fluke/*H. pylori* co-infection is the central player in biliary

disease manifestations including CCA in opisthorchiasis in northeastern Thailand. However, the underlying mechanisms by which *H. pylori* associates with the liver fluke, opisthorchiasis and CCA remain to be established.

4. Pathogenesis of *H. pylori* induced biliary diseases

Pathogenesis and disease outcomes following infection with *H. pylori* are mediated by a complex interplay between bacterial virulence factors, host, and environmental factors. Unfortunately, only a few studies describe the pathogenesis in *H. pylori* induced biliary diseases [26, 28, 30]. In brief, following entry of *H. pylori* into host tissue, four steps are critical for the bacterium to establish successful colonization, persistent infection, and disease: 1) survival in environment (the acidic stomach or the alkaline bile ducts); 2) movement toward epithelial cells by flagella-mediated motility; 3) attachment to host cells mediated by interactions between bacterial cell adhesins and host cell receptors; and 4) tissue damage following the release of toxins, specifically CagA and VacA [34, 35].

5. Survival of *H. pylori* in the bile

H. pylori and other species of *Helicobacter* can survive at the alkaline pH of the bile as they can be detected in bile sampled from biliary diseases including CCA [36, 37]. Most of the *H. pylori* and other bacteria in the bile are coccoid form that may reflect responses to bile acids [38]. In addition, biofilm formation by the bacteria in the bile seems facilitate survival in the environment within the human biliary tract [36]. Interestingly, an increased abundance of *H. pylori* virulence genes, i.e. *cagA* and *vacA* was observed in extrahepatic CCA compared to benign biliary diseases [37]. Similarly, significant higher frequencies of *cagA*-positive *H. pylori* have been reported during CCA than cholelithiasis and in bile from healthy individuals [26]. Biliary micro-environmental components such cholesterol may enhance the pathogenicity of *H. pylori* as it acquires host cholesterol for catabolism of lipopolysaccharide (LPS) for cell membranes [39]. In addition, cholesterol promotes growth of *H. pylori* in serum-free media [40]. Together, these findings provide support to the notion that *H. pylori* can be more virulent within the biliary environment.

6. *Opisthorchis* is a reservoir of *H. pylori* and host-bacterial interaction

We recently reported that *O. viverrini* was a reservoir of *H. pylori* (Figure 1) [31]. The intensity of infection with *H. pylori* within *O. viverrini* infected hamsters was significantly greater than that of uninfected hamsters (Figure 2). The *H. pylori* bacteria localized on the gut epithelium of fluke and survived in the adult liver flukes *in vitro* co-cultured with antibiotics for more than 30 days [31]. These findings indicate the establishment of *H. pylori* benefits from the support of the liver fluke gut. As the pH in the secretions from the gut of *O. viverrini* is approximately pH 5–6, compared to the pH 7–8 of the bile, perhaps the liver fluke protects the *H. pylori* bacilli from the otherwise inimical environment of the bile. Beyond survival in environmental milieu and pH of the gut of the liver fluke within the lumen of the bile duct, establishment of infection by *H. pylori* requires adhesion and colonization of the gut epithelium by *H. pylori* to protect the bacilli from displacement from the gut by forces such as those generated by peristalsis and emptying similar to those

described in the mammalian stomach. Specific interaction between the bacterial adhesins and the host (fluke) gut epithelium receptors is needed.

There are several adhesins in *H. pylori* including blood-antigen binding protein A (BabA) and sialic acid-binding adhesin (SabA), neutrophil-activating protein (NAP), heat shock protein 60 (Hsp60), adherence-associated proteins (AlpA and AlpB), *H. pylori* outer membrane protein (HopZ), and lacdiNAc-binding adhesin (LabA) [35] that interact with host tissues and cells. BabA mediates binding of the bacteria to Lewis B antigens, Le^b [41] and related terminal fucose residues found on blood group O (H antigen), A and B antigens [42]. Similar to BabA, the SabA has affinity for sialyl-Le^x [43]. Toll-like receptor 4 (TLR4) is a known receptor for bacterial LPS [44]. The LabA adhesin specifically binds to GalNAcβ1-4GlcNAc motif, also known as N,N′-diacetyllactosediamine [lacdiNAc]), carried by MUC5AC mucins [45, 46]. However, orthologues of the human cell receptors and antigens have yet to be described from the epithelium of the gastrodermis of the gut of *O. viverrini*. We have begun laboratory investigation these aspects, with initial findings that support the role of liver fluke as a reservoir of *H. pylori*.

7. *Helicobacter pylori* – biliary epithelium interaction

Concerning the gastric epithelium, *H. pylori* colonizes on the mucosal layer, and adheres to the epithelium through interactions between bacterial adhesins with cellular receptors (as described above) [35], after which virulence factors stimulate cascades of inflammatory signalling, anti-apoptosis, cell proliferation and transformation pathways [18, 19]. Moreover, *cag*A-positive *H. pylori* has been shown to induce mutation of the gastric carcinoma cell line [47]. However, only a few reports have described the interaction between biliary epithelium and *H. pylori*. Our previous studies revealed that *H. pylori* induces multiple effects in CCA cell lines *in vitro*, including inflammation (IL-8 production), cellular proliferation and apoptosis [28, 30]. In addition, at a low multiplicity of infection (MOI=1), *H. pylori* induces pro-inflammatory cytokine production and proliferative responses in CCA cell lines. These findings suggest that the small numbers of *H. pylori* bacteria that reach the biliary epithelial cells suffice to promote inflammation and transformation within this niche. Hence, the findings provide support for the potential role of this carcinogenic microbe in the development of hepatobiliary disease [28]. In order to investigate this hypothesis, as well as links between *cag*PAI-positive *H. pylori* strains and CCA, we tested the ability of various *H. pylori* wild-type and isogenic *cag* mutant strains to adhere and induce pro-inflammatory responses in two CCA cell lines [29]. All of the strains adhere to both CCA cell lines without significant differences among the different virulence factors, and in addition, *H. pylori* wild-type bacteria stimulated significantly higher responses in all cell types compared with *cag*A$^-$, *cag*L$^-$ or *cag*PAI$^-$ strains. Furthermore, *H. pylori* requires a functional type 4 secretion system (T4SS) for the activation of NF-κB, leading to the production of IL-8, in biliary tract epithelial cells [41]. Together, these several reports reveal that the *cag*PAI is critical for *H. pylori*–related pathogenesis in biliary epithelia, and thus provide a potential causal link for *H. pylori* in biliary tract disease including CCA.

8. Liver fluke enhances *H. pylori* colonization and adhesion to biliary epithelium?

Since *O. viverrini* has a blind gut (like all trematodes it does not have an anus), all ingested materials in the gut including bacteria are regurgitated out through the oral sucker. To date there are no reports on the link between *O. viverrini* infection and enhancement of *H. pylori* colonization. However, we have reported the up-regulation of TLR 4 but not other TLRs in normal cholangiocytes (H69) co-cultured with *O. viverrini* excretory-secretory products [48]. As TLR 4 is a known receptor for *H. pylori*, this result may imply the possible increased adhesion on biliary epithelium in *O. viverrini* infection. Other host receptors for *H. pylori* also may be involved in the colonization. Indeed, colonization of *Helicobacter* in mucosal layer needs its sheathed flagella to move across the mucus layer to the epithelium [35]. Given *O. viverrini* is rather large in size (up 1 cm in length in the human biliary tract) and can mechanically damage the mucous layer of the biliary epithelium, and thus facilitate bacterial adhesion.

9. Pathogenesis of liver fluke/*Helicobacter* induced pathology and carcinogenesis

Three main mechanisms are proposed to contribute to CCA through chronic infection with *O. viverrini*: mechanical damage to the biliary epithelia caused by the feeding activities of the parasites, immunopathology due to infection-related inflammation, and toxic effects of parasite ES. The interplay of these mechanisms aligns with current understanding of this malignancy, suggesting formation and progression relies on many interrelated factors creating a microenvironment that is conducive for malignant transformation [49]. However, these interplays may not explain or entirely explain the role of *O. viverrini*. Additionally, the influence and activities of *H. pylori* transported by *O. viverrini* into the biliary tract may be central to pathogenesis and carcinogenesis of liver fluke-associated CCA.

9.1. Mechanical injury

Mechanical injury caused by the liver flukes contributes to biliary damage. The suckers of the fluke attach to biliary epithelia, damage the bile ducts, even in early infection. As the flukes mature, the lesions enlarge and ulcerate. The ulcers provide a portal of egg entrapment, inducing circumoval granuloma during chronic opisthorchiasis that in turn leads to biliary periductal fibrosis [2]. Along with inflammation, the ulcer allows facilitates entry of for bile acids and other bile constituents, exposure to which can predispose to malignant transformation given these metabolites represent endogenous etiologic agents in gastrointestinal cancers [50]. In addition, migration of the liver flukes within the bile ducts and their grazing activity in mucin along the biliary epithelium ensures close contact between *H. pylori* and the cholangiocytes lining the bile ducts.

9.2. Metabolic products

Liver flukes release excretory/secretory products (ES) from the tegument and excretory openings into the bile, or culture medium *in vitro*, some of which are highly immunogenic

[51, 52]. Recent findings revealed that *O. viverrini* ES products contained *H. pylori* [31]. These ES products, aside from inducing immune responses, may be toxic to or interact with the biliary epithelium [53]. Murine fibroblasts (NIH-3T3) co-cultured with *O. viverrini* (but physically separated from the worms in Transwell plates) proliferate compared to cells in media alone [54]. Human biliary cells also proliferate in the presence of ES-derived parasite growth factors, i.e. granulin [55] and anti-apoptotic proteins [56]. *H. pylori* itself can promote cell proliferation and transformation and anti-apoptosis as noted above. These data together demonstrate clearly that metabolic products of *Opisthorchis viverrini*, which includes *H. pylori* or its components, orchestrate the induction of cell proliferation and anti-apoptosis, confirming pioneering reports of hyperplasia of opisthorchiasis-associated biliary epithelial cells [57, 58].

9.3. Immunopathology

Host immune responses and immunopathological processes mediate hepatobiliary damage in opisthorchiasis [57–60]. We have implicated parasite-specific IL-6 in the pathogenesis of advanced periductal fibrosis in opisthorchiasis, with links to other hepatobiliary abnormalities, including CCA [61]. Sripa and Kaewkes [57] showed that inflammation around infected hamster bile ducts was a consequence of host cellular responses to antigens of *O. viverrini*. Marked infiltration of inflammatory cells in periductal sites of infected liver was associated with the presence of fluke antigens in the bile duct epithelium as detected by immunohistochemistry (IHC). Intense antigen staining was seen adjacent to the flukes. Small bile ducts, the secondary/third order ducts - where flukes do not occur because the diameter of the ducts is not sufficiently wide to accommodate parasites – also were positive for *O. viverrini* antigens and were markedly inflamed. By comparison, *H. pylori* also triggers pro-inflammatory cytokine responses by biliary cells [29] that can cause inflammation in infected hosts. *O. viverrini* ES products of the liver flukes, which may contain *H. pylori* components, induce IL-8 production that is unique cytokine for *H. pylori* in normal cholangiocytes [48]. Therefore, we predict that both *O. viverrini* and *H. pylori* synergistically induce pathologies through immunopathogenic process in the infected biliary tract.

10. Multi-factorial pathway from *O. viverrini*/*H. pylori* infection to CCA

Figure 3 pictorially indicates the several mechanisms that have been proposed to explain how infection with *O. viverrini* provokes cholangiocarcinogenesis. The primary pathologic change, i.e. epithelial desquamation and ulcer, may be due to mechanical irritation caused by the liver fluke, *H. pylori* and/or the ES released by the liver flukes. However, as outlined above, immunopathological processes contribute to the longstanding hepatobiliary damage. With liver fluke and *H. pylori* co-infection, inflammation, periductal fibrosis and proliferative responses including epithelial hyperplasia, goblet cell metaplasia and adenomatous hyperplasia represent predisposing lesions that may enhance susceptibility of host cell chromosomal DNA to genotoxic lesions [4, 62]. *N*-nitroso compounds and their precursors occur at low levels in fermented food, including fermented fish, e.g., *plara*, which is ubiquitous in the diet of people in much of Thailand and neighboring Laos. Indeed, it has

been argued that these compounds are requisite along with liver fluke infection as carcinogens leading to CCA in the inhabitants of these regions [63].

11. Conclusion

Infection with *Opisthorchis viverrini* is a cogent risk for cholangiocarcinoma. Thailand has the highest rates of both opisthorchiasis and cholangiocarcinoma in the world. Moreover, in this location, the prevalence and geographical range of carriage of *Helicobacter pylori* parallels those of opisthorchiasis and cholangiocarcinoma. Our review on recent literature reveals that the liver fluke is a reservoir of *Helicobacter* spp. and addresses the role of liver fluke associated *H. pylori* in hepatobiliary disease and malignancy. We hypothesize that co-infection by *O. viverrini* and *H. pylori* is central of liver fluke infection associated cholangiocarcinoma. Researches on several aspects of the two carcinogenic pathogens co-infection are currently carried out in our laboratory.

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Highlights

- *Opisthorchis*/*Helicobacter* co-infection is central to opisthorchiasis
- *Opisthorchis* is a reservoir of *H. pylori*
- *H. pylori* may play key role in pathogenesis of opisthorchiasis-induced cholangiocarcinoma

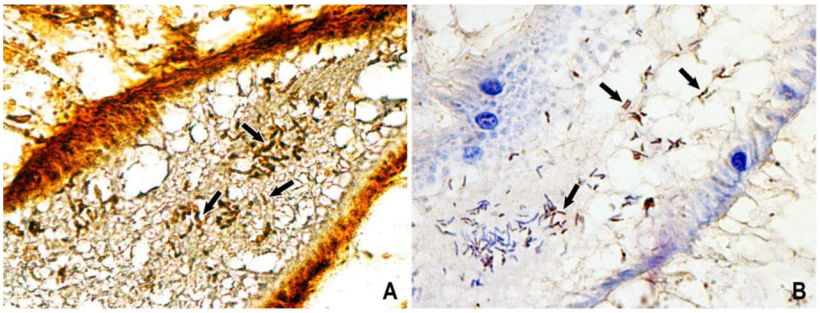

Figure 1.

Identification of *Helicobacter pylori* in the gut of *Opisthorchis viverrini*. (A) Cluster of *H. pylori*-like (short arrow) stained with the Warthin-Starry method shown as dark brown curved rod-like bacteria in the lumen. (B) Specific detection of *H. pylori* with anti-*H. pylori* antibody immunostained as a dark brown color (short arrow). Original magnification, x400. (From Deenonpoe et al (2015) *Asian Pac J Cancer Prev* 18, 1751, with permission.)

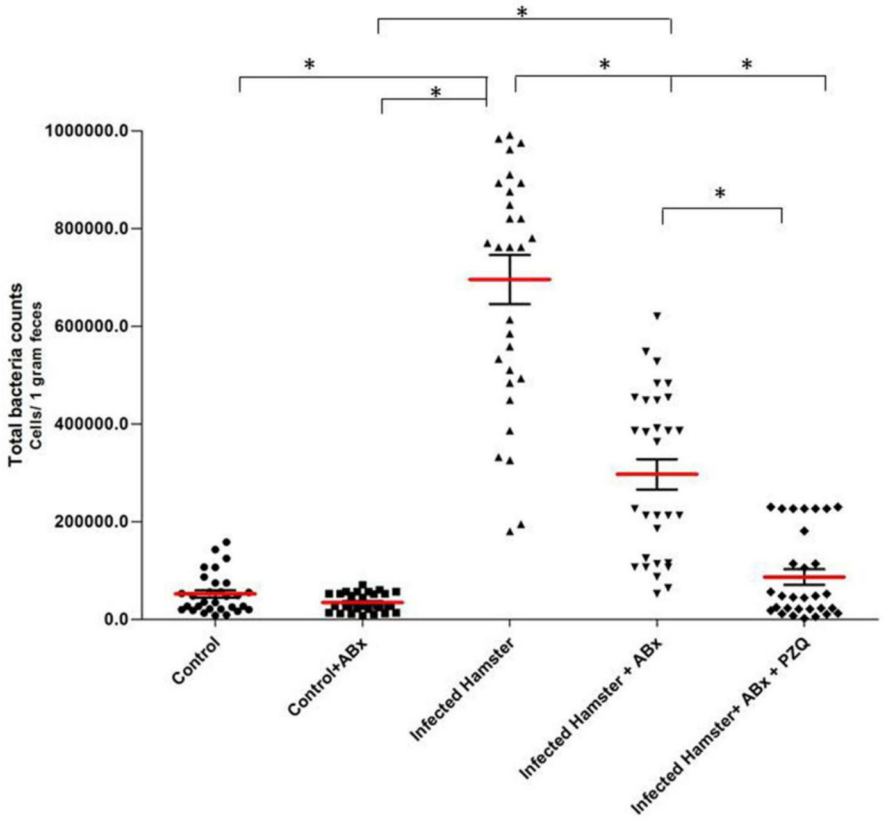

Figure 2.

Quantification of *Helicobacter pylori* in colorectal feces of hamsters using quantitative RT-PCR. Total bacterial cell counts among the following 5 groups of hamsters are compared; 1. Control (Group 1), 2. Control+ ABx (Group 2), 3. Infected hamster (Group 3), 4. Infected hamster + ABx (Group 4), and 5. Infected hamster + ABx+ PZQ (Group 5). * Significant differences among groups, $P \leq 0.001$. (From Deenonpoe et al (2015) *Asian Pac J Cancer Prev* 18, 1751, with permission.)

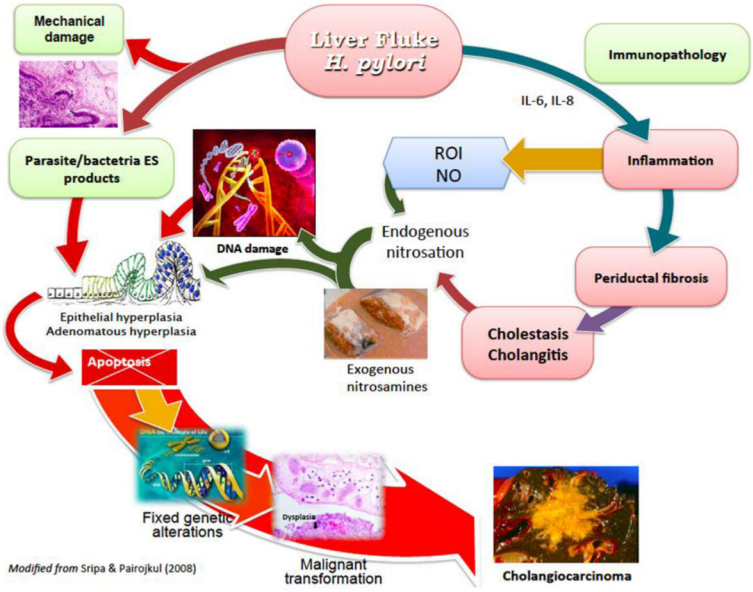

Figure 3. Hypothesized pathways of pathogenesis of opisthorchiasis/*H. pylori*-induced cholangiocarcinoma

The liver fluke *Opisthorchis viverrini/Helicobacter* damages bile duct tissue via at least three distinct pathways: 1) mechanical damage to biliary epithelia caused by parasites sucking; 2) inflammation-induced immunopathology, particularly due to reactive oxygen intermediates (ROI) and nitric oxide (NO*); and* 3) direct effects of fluke/Helicobacter secreted proteins on biliary epithelia including cell proliferation induced by parasite-derived growth factors. These pathways converge, resulting in genetic lesions and unregulated proliferation. Damaged DNA/genes after successive replications become fixed, leading to malignant transformation of cholangiocytes. Adapted from [32].