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Infection with the carcinogenic human liver fluke, *Opisthorchis viverrini*

Michael J. Smout[1], **Banchob Sripa**[2], **Thewarach Laha**[3], **Jason Mulvenna**[1], **Robin B. Gasser**[4], **Neil D. Young**[4], **Jeffrey M. Bethony**[5], **Paul J. Brindley**[5], and **Alex Loukas**[1]

[1]Queensland Tropical Health Alliance, James Cook University, Cairns, QLD, Australia
[2]Department of Pathology, Khon Kaen University, Khon Kaen, Thailand [3]Department of Parasitology, Khon Kaen University, Khon Kaen, Thailand [4]Department of Microbiology, Immunology and Tropical Medicine, George Washington University, Washington DC, USA
[5]Department of Veterinary Science, The University of Melbourne, VIC, Australia

Summary

Throughout Southeast Asia there is a strikingly high incidence of cholangiocarcinoma (CCA - hepatic cancer of the bile duct epithelium), particularly in people from rural settings in Laos and Northeast Thailand who are infected with the liver fluke, *Opisthorchis viverrini*, one of only three carcinogenic eukaryotic pathogens. More ubiquitous carcinogenic microbes, such as *Helicobacter pylori*, induce cancer in less than 1% of infected people, while as many as one-sixth of people with opisthorchiasis will develop CCA. The mechanisms by which *O. viverrini* causes cancer are multi-factorial, involving mechanical irritation from the activities and movements of the flukes, immunopathology, dietary nitrosamines and the secretion of parasite proteins that promote a tumourigenic environment. Genomic and proteomic studies of the liver fluke secretome have accelerated the discovery of parasite proteins with known/potential roles in pathogenesis and tumourigenesis, establishing a framework towards understanding, and ultimately preventing, the morbidity and mortality attributed to this highly carcinogenic parasite.

Carcinogenic helminths

More than 340 species of helminths infect humans, of which at least 25 cause serious and occasionally fatal outcomes [1]. Currently, only three of these helminths, *Schistosoma haematobium* (blood fluke), *Clonorchis sinensis* (Chinese liver fluke) and *Opisthorchis viverrini* (Thai liver fluke), have been definitively linked to cancer (Table 1) [2]. It has been suggested that over a lifetime almost one in six of *O. viverrini* infected individuals will contract cholangiocarcinoma (CCA), or cancer of the bile ducts [3, 4]. CCA is a highly aggressive cancer with a very poor prognosis [5, 6]. Despite this, flukes contribute only minimally (0.12%) to world cancer rates. One major reason is simply the limited distribution of these parasites. As with many parasitic helminths, liver flukes tend to be restricted geographically due to their reliance on specific intermediate hosts (freshwater snails and fish) for propagation and transmission (Figures 1 and 2). This limits endemic areas to tens of millions, rather than the billions of individuals inhabiting regions rife with viral infections [7]. Moreover, with *O. viverrini* cultural habits play a critical role in transmission and subsequent restriction of endemic regions (see below). Another contributing factor to the small proportion of fluke-derived cancers is the difficulty in identifying (unambiguously) correlations between infections with multicellular organisms and cancer, particularly in developing countries, and as a result, current estimates are conservative [4]. It has been asserted that the incidence of *O. viverrini*-derived cancer may be six times greater than otherwise estimated [4]. Other studies suggest that *O. viverrini* infection may initiate as many

as 89% of the 28,000 annual liver cancer cases in Thailand, making the infection the fourth largest cause of death in Thailand behind AIDS, stroke and traffic accidents [8, 9].

While *O. viverrini* has been recognised as a group I carcinogen since 1994, its close relative *C. sinensis* has only recently been classified within group I [2]. The CCA rates in Korea, a major region of *C. sinensis* endemicity, ranges from 7.6–12 ASR (age standardised ratio); while high compared to the rest of the world (<5 ASR) these values are low compared to areas in North-East Thailand where *O. viverrini* is endemic, with a CCA rate of 58.1 ASR [10, 11]. The reasons for the substantially lower ASR in Korea are unclear. Additional data might also elevate the status of the lesser researched Siberian liver fluke, *Opisthorchis felineus,* to group I carcinogen status, where *O. viverrini* and *C. sinensis* are currently ranked [2, 12].

While investigations have been limited, 12 reports over the last 20 years describe potential links between other parasitic worms and carcinogenesis – some of these studies describe soluble parasite extracts that cause DNA damage and mitogenesis, such as the liver fluke of livestock *Fasciola hepatica*, *Taenia* spp. tapeworms and various nematodes [reviewed in [13]]. Behind *O. viverrini* however, the most widely accepted carcinogenic helminth is the blood fluke *S. haematobium*. Of the human schistosomes, only *S. haematobium* has been unequivocally linked to cancer and is a group I carcinogen [12]. Adult worms reside in the vasculature of the urinary bladder where the female fluke lays eggs which penetrate the bladder wall to be passed in the urine. Some eggs are trapped in the tissue by granulomatous immune reactions, resulting in chronic fibrosis over time. In Egypt, where *S. haematobium* infection is common, bladder cancer is the most prevalent of all cancers in males compared with the fifth to seventh most common cancer in countries where schistosomiasis is not endemic [reviewed in [14]]. Squamous cell carcinoma (SCC) is the most frequently encountered subtype of bladder cancer associated with *S. haematobium*, while transitional cell carcinoma is the dominant form in non-endemic areas. A role for nitrosamines (urinary) in *S. haematobium* has been proposed to predispose infected individuals to SCC [15]. As with opisthorchiasis associated CCA (see later), there is a proposed role for *S. haematobium* soluble proteins in the development of SCC. *S. haematobium* adult worm extract caused CHO cells to undergo increased proliferation and decreased apoptosis, accompanied by down-regulation of tumour suppressor p27 and up-regulation of the anti-apoptotic B-cell lymphoma -2 protein (Bcl-2). Moreover, cells exposed to parasite proteins displayed increased migration and invasion capabilities [16], and formed sarcomas when injected into male nude mice [17]. For a comprehensive recent review on the role of *S. haematobium* in carcinogenesis, see [14].

Liver flukes, with a specific focus on *Opisthorchis viverrini*

To date 13 species of liver flukes have been recovered from humans, but only four are commonly encountered – *F. hepatica, C. sinensis, O. viverrini* and *O. felineus.* Worldwide infections with liver flukes are estimated at over 45 million, predominantly across Asia and Eastern Europe [reviewed in [18]]. The most pathogenic of these flukes is *O. viverrini,* which infects 10 million individuals throughout Thailand, Laos, Cambodia and Vietnam [7]. *C. sinensis* infects over 35 million individuals across a far wider area than *O. viverrini*, encompassing Korea, mainland China, Taiwan, Vietnam, probably Thailand and formerly Japan [18, 19]. *F. hepatica* is estimated to infect 2.4 million people around the world, and *O. felineus*, primarily a parasite of cats, infects only 1.2 million people in Eastern Europe and the Russian Federation [20–23].

O. viverrini has three hosts, two fresh water intermediate hosts (a pulmonate snail and a cyprinoid fish) and a primary mammalian host, usually humans, but can also be fish-eating

mammals such as domesticated cats and dogs (Figure 2). Still, these alternate primary hosts are largely insignificant as carriers in endemic areas, as low prevalence is observed, and their egg output is considered a minor contributor to the maintenance of the life cycle relative to that of humans [24]. Unlike the many soil transmitted helminths, which often directly infect the mammalian host through the skin, *O. viverrini* is food-borne and is infective when a viable metacercaria is ingested. Hence, regions where they are endemic are not only limited by availability of intermediate hosts, but also to areas where people routinely consume uncooked and under-cooked fish. These constraints reflect the striking regional distribution between the four major geographical regions within Thailand, with only the North-East and Northern regions suffering from appreciable levels of infection (19% and 15.7% prevalence, respectively). It should be noted that North-East Thailand has the highest prevalence of infection with *O. viverrini* but it also has extensive mass anthelmintic drug administration programs; before these were widely implemented the region had an infection rate of 34.6% [8, 25].

After ingestion by the mammalian definitive host, the metacercariae excyst in the duodenum with the help of the host's digestive enzymes. They then migrate up the common bile duct to the intrahepatic bile duct branches where they mature into dorso-ventrally flattened, leaf shaped adult worms of approximately 7 × 1.5 mm in size. It takes about one month from ingestion of metacercariae to the detection of eggs in the human faeces. The exact life span of adult worms is unknown, but is thought to be at least 10 years and possibly 25–30 years [26]. Some enzyme-immunoassays have been developed for diagnosis of past and present infections but suffer from lack of specificity and so currently observation of eggs in the faeces is the best way to determine current infection status [7, 8, 21, 27, 28].

Liver cancer and Opisthorchis viverrini

Primary liver malignancies

The majority of primary malignant liver cancers are of two main histological types distinguished by their cellular origin. First, hepatocellular carcinoma (HCC) is derived from hepatocytes and is the most common form throughout the world [2]. There are several important risk factors for HCC, with 75–80% of cases attributable to hepatitis B and C infection [29, 30]. Second, CCA is derived from cholangiocytes, which form the epithelial lining of the bile ducts. CCA is by far the least common of the two cancers, except for regions within East Asia where opisthorchiasis and/or clonorchiasis are endemic. These regions show a 15-fold increase in the liver cancer incidence compared to the incidence of this cancer in the developed world (71.9 vs <5 ASR) and 50–100 times the CCA incidence (58.1 vs <1 ASR) [11]. As previously mentioned, *C. sinensis* causes significant CCA in Korea, but at rates more than 5 times lower (58.1 vs 7.6–12 ASR) than those seen in North-East Thailand [2].

Both CCA and HCC have a low survival rate with a yearly fatality ratio of approximately 1.0, which indicates for the most part that patients do not survive one year past diagnosis [8]. Even with treatment, the five year relative survival rates during the 1990s in the United States and Europe were only 8.3% and 6.5% respectively [31, 32]. In developing countries, prognosis with liver cancer usually is poor.

Mechanisms of *Opisthorchis viverrini*-initiated CCA

Tumours are rare in infections with carcinogenic viruses and bacteria

The viral modification of cell growth resulting in cancer has been extensively studied and was vital in establishing many current concepts of cancer biology. Research on viruses has shown that the malignant phenotype, in most cases, is the end result of a series of genetic

and epigenetic changes rather than a single event. The likelihood of these aberrant alterations synchronising in a single cell, even upon exposure to a carcinogen, is low due to the complex, multi-faceted nature of the phenomenon. One common example illustrating this point is *H. pylori*. Half the human population is infected with this gram positive bacterium but less than 1% will proceed to develop the associated gastric cancer and/or Gastric Mucosa–Associated Lymphoid Tissue (MALT) Lymphoma [33]. By contrast, and notwithstanding controversy on the exact proportion of cancers attributable to *O. viverrini*, it is suspected over a lifetime of infection that as many as one in six infected people will develop CCA [3, 4].

Proving an infectious agent is carcinogenic is a challenge, but exposing all or even some of the mechanisms involved is an extremely complicated process, especially where parasitic worms are concerned. Infected populations usually suffer from multiple parasitic infections and other medical conditions and risk factors that confound the analyses. Additionally, there have recently been cases of *C. sinensis* infections in central Thailand villages, detected by PCR-based methods [19]. As the diagnosis of infection is by detection of eggs in faeces and both *C. sinensis* and *O. viverrini* eggs are morphologically very similar, it is unknown how widespread *Clonorchis* may be throughout Thailand. This distinction between parasites may be crucial for ongoing studies involved in widespread screening for *Opisthorchis* infection and liver abnormalities or cancer [4].

Proposed mechanisms contributing to liver fluke-induced carcinogenesis

Three main mechanisms are proposed to contribute to CCA through chronic infection with *O. viverrini*: (a) mechanical damage to the biliary epithelium caused by feeding parasites; (b) immunopathology due to the inflammatory effect of the infection; (c) toxic effects of parasite secreted molecules (Figure 3). The interplay of these three mechanisms aligns with current knowledge of malignancies, suggesting formation and progression relies on many interrelated factors creating a microenvironment that is conducive for malignant transformation [34].

Mechanical damage induces a wound response

The body's response to wounding—Wound repair has long been implicated in tumourigenesis with striking similarities between tumour stroma and the wounded tissues [35]. This carcinogenic potential of wound repair was first demonstrated in the 1980s. An adult chicken injected with the carcinogenic respiratory syncytial virus developed a tumour solely at the infection site even though the virus spread completely throughout the body [36]. After this initial malignancy, any subsequent wounding of an infected bird was sufficient to elicit new carcinogenic growth at the injury site. These wound healing responses disrupt the cellular microenvironment by inducing production of enzymes, including matrix metallo- and other proteases, that degrade the extracellular matrix, interfere with epithelial cell-cell adhesion and induce cell division to create a cellular covering of the wound [34, 37]. In a normal situation, after the wound has been repaired, a series of secreted factors act to halt the process.

How does this apply to liver flukes?

Undoubtedly liver flukes cause mechanical injury to the bile ducts, easily visualised as tissue damage from the oral and ventral suckers hooking into the bile duct epithelium to secure the parasite in place (Figure 4) [38]. If the parasite is removed the damage is repaired and the regenerative growth of the wound response halts. But in a fluke infection, persistent damage of the epithelium resulting from feeding and migratory activities hinders complete wound repair and recovery from the injury. The constant cell division associated with this mechanically driven tissue injury, in the presence of exogenous co-factors, such as dietary

nitrosamines (see later), is thought to result in DNA damage and subsequent oncogenesis, as indicated in Figure 3. These carcinogenic properties of fluke-induced wounding have been illustrated in hamsters, where the bile ducts were surgically ligated, simulating damage seen in fluke infections, followed by co-administration of sub-carcinogenic doses (20 mg/kg) of nitrosamines. The hamsters showed significant biliary lesion development whereas the controls, which received biliary ligation or nitrosamines alone, did not [39]. Therefore, invoking the wound repair response likely triggers cell proliferation, which in the presence of co-factors, significantly contributes to cancer development [40].

Immunopathological changes

An intense infiltration of inflammatory cells in infected livers has been associated with *Opisthorchis* antigens in the bile duct epithelium, observed by immunohistochemical staining of liver sections from experimentally-infected hamsters [41]. This intense inflammatory response can result in malignancy in a number of ways - directly through production of oxidative radicals (discussed further below) or indirectly by influencing DNA replication which creates a phenotype that is predisposed to mutations [42]. In other pre-cancerous conditions, such as ulcerative colitis, chronic inflammation has been shown to produce a 'mutator' phenotype, inducing conditions conducive to microsatellite instability and erroneous DNA repair [43].

Early inflammatory events predominantly comprise the recruitment of neutrophils, eosinophils and macrophages, whereas later stage infections show the major infiltrating cells are mononuclear subtypes including lymphocytes, macrophages, plasma cells and mast cells [44]. In addition to the fluke invoking a localised response, the excretory/secretory (ES) molecules released by the parasite induce inflammation in the first order bile ducts which are too small for flukes to reside in [41]. Despite these findings, it is still unknown whether these released antigens are entrapped by the biliary epithelium via a receptor mediated or a general endocytotic process. Notably, a host genetic component may be involved, as not all infected hamsters exhibit parasite antigens on biliary epithelial surfaces [41, 44].

In humans infected with *O. viverrini*, inflammatory changes to the liver can be detected early during *O. viverrini* infection before chronic disease sequelae are apparent. In a recent case-control study, we determined the presence of advanced periductal fibrosis (APF) in asymptomatic, *O. viverrini*-infected individuals and then measured cytokine responses to *O. viverrini* ES proteins. In persons with APF, levels of the pro-inflammatory cytokine interleukin-6 (IL-6) to *O. viverrini* ES products were 8-fold higher than levels in *O. viverrini* infected individuals without APF [45]. Moreover, elevated IL-6 was associated with increased risk of APF by 63% in a model adjusted for sex and age. *O. viverrini*-infected individuals with APF showed other hepatobiliary abnormalities, including reduced gall bladder contractility. Taken together, these data strongly implicate a role for parasite-specific IL-6 in the pathogenesis of APF in opisthorchiasis.

Chronic immune responses can lead to cancer

Activated phacogytes produce bursts of nitric oxide (NO) and oxygen radicals which normally destroy many pathogens [46]. This immune defence mechanism, however, has potentially inimical side-effects - NO is mutagenic and results in the release of endogenous carcinogenic nitrosamines, one of the key initiators of CCA (Figure 3) [47]. Unfortunately, for the host, when the invading pathogen is unaffected by these inflammatory responses, such as with *O. viverrini,* a prolonged reaction and associated NO accumulation develops [48–50]. Fluke infections in humans results in elevated plasma and urinary nitrite levels, even in the absence of elevated levels of dietary nitrosamines and their precursors [50, 51]. This rise in nitrites indicates high endogenous nitrosamine production, as nitrites are the excretory

metabolites of nitrosamines. This consequence of fluke infection is reversible upon removal of the parasite, which is readily accomplished by treatment with the anthelmintic drug, praziquantel [52]. Follow-up investigations proved that DNA damage from nitrite production is present at the sites of fluke-induced carcinogenesis [53]. Further evidence implicating nitrosamines in the pathogenesis of opisthorchiasis comes from studies linking urinary nitrite levels, infection intensity and an increased risk of CCA [3]. It is unclear if the increased nitrite levels are the result of elevated exogenous nitrites from fermented foods, such as fish sauce, or differences in host endogenous nitrite production.

Another source of prolonged inflammation is not directly from the flukes but the eggs they produce. Eggs released from the fluke become entrapped as a consequence of bile duct blockage or embedded within the periductal tissue through ulceration at the site of parasite attachment [39]. Significantly, this egg related inflammation of surrounding tissue can operate long after the adult flukes have been expelled by chemotherapy with praziquantel. This carcinogenic potential of *O. viverrini* eggs has not been studied in depth but we suggest that it may significantly contribute to carcinogenesis as seen with the other major carcinogenic fluke, *S. haematobium* [54].

Exogenous nitrosamines: critical for carcinogenesis?

Nitrosamines are common in the fermented foods that are a dietary staple in North East Thailand [28]. When ingested in large quantities, such as in fermented fish, nitrosamines are carcinogenic [55]. Short term infection (under 7 months) of hamsters with *O. viverrini* alone does not commonly lead to cancerous lesions, however, substantial numbers of lesions are induced with the addition of normally sub-carcinogenic doses of nitrosamines to the diet of infected hamsters [39, 56]. It should be noted that longer infections (20 months) have shown that *O. viverrini* alone is sufficient for CCA induction [57]. While the exact role of nitrosamines in human fluke infections is unclear, regular consumption of fermented fish containing metacercariae poses an added risk for CCA.

Mitogenic properties of fluke ES products

To survive long periods in hostile environs, parasitic helminths excrete and secrete a range of soluble proteins and other mediators that perform many roles at the host-parasite interface, including digestion of nutrients, tissue invasion and regulating the host immune system. This interaction has long been thought to modify host cellular homeostasis and contribute to malignant transformations [58, 59]. Secretions of other infectious agents have supported this notion: *H. pylori* causes cell proliferation through protein transfer, and schistosome secretions and tapeworm soluble products induce hyperplasia [60–63]. A routine observation in fluke infections is altered cell states, one of the gateways to genomic instability that can be induced by a variety of growth factors [64]. Lesions of epithelial dysplasia and metaplasia including goblet cell metaplasia and adenomatous hyperplasia are commonly seen [65]. Fluke associated CCA cases frequently demonstrate an intestinal goblet cell phenotype which differs from non-infection related CCA [66]. *O. viverrini* ES products are mitogenic and taken up by host biliary cells (discussed below), and *in vitro* application of ES products to mammalian cell lines induces morphological changes [67]. Subsequent *in vivo* investigations confirmed that CCA development in the hamster model of opisthorchiasis is more rapid when hamsters are administered with ES products prior to *O. viverrini* infection (B. Sripa, unpublished), suggesting that immunological memory might further accelerate tumourigenesis.

O. viverrini ES products induce cell proliferation

Using a non-contact *in vitro* co-culture technique, *O. viverrini* ES products were shown to induce proliferation of non-cancerous fibroblasts, a keystone of cancerous transformation [67].

This insightful experiment involved co-culturing of *O. viverrini* adult worms with NIH-3T3 mouse fibroblasts physically separated by an 8 μm membrane, thereby removing mechanical (and immunological) damage from the interpretation of the outcome of the experiment. Three-fold growth of cells compared to control cells cultured in the absence of parasite proteins was observed, confirming that ES products of *O. viverrini* contain growth factors or other molecules that result indirectly in cell growth (and potentially contribute to the development of CCA). Excessive cell growth resulted from stimulation of cells at the G1/S transition in the cell cycle and was induced by over expression of cyclin D1 and phosphorylation of retinoblastoma protein [67].

The effect on signalling of mouse fibroblasts after exposure to *O. viverrini* ES products was explored using complimentary DNA microarrays. More than 200 genes underwent significantly elevated expression upon addition of ES products [68], two of which had known roles in cell proliferation - *eps* 8, or epidermal growth factor (EGF) receptor pathway substrate, and *tgf*-β 1*i*4, or transforming growth factor-β-1 (TGF-β)-induced transcript 4. The TGF-β super family generally act as tumour suppressors by blocking cell cycle progression at the G1 stage, but TGF-β is more often linked to later stages of cancer progression [69]. EGF is also a large gene family with links to proliferation, and errant activation of EGF signalling pathways can result in cancer [70].

The transcriptome and secreted proteins of *O. viverrini*

Recently, the transcriptomes of *O. viverrini* and *C. sinensis* were sequenced using high throughput sequencing. Approximately 50,000 sequences were assembled for each species, defining molecules that are essential for the development, reproduction and survival of these flukes [71, 72], including a number of secreted growth factors which might act on host cells [73]. This extensive dataset provided the framework for characterisation of the secretome using proteomic approaches [74]. To identify parasite proteins critical for liver fluke survival and the aetiology of CCA, various proteomic approaches were employed to characterise 300 *O. viverrini* secreted and surface membrane proteins. The ES products included a complex mixture of proteins that have been associated with cancers, including proteases, protease inhibitors, orthologues of mammalian growth factors and anti-apoptotic proteins. Of particular interest was the identification of *Ov*-GRN-1, a protein with sequence similarity to the mammalian growth factor, granulin.

Granulin: a parasite growth factor that causes proliferation of host cells

Ov-GRN-1 is the only helminth-derived growth factor reported to date that causes proliferation of mammalian cells [75]. *Ov*-GRN-1 is secreted by adult flukes where it binds to and is internalised by biliary epithelial cells of *O. viverrini*-infected hamsters (Figure 5). Recombinant *Ov*-GRN-1 caused mouse fibroblasts and human CCA cell lines to proliferate via the mitogen-activated protein kinase (MAPK) pathway, and anti-*Ov*-GRN-1 antibodies blocked the mitogenic activity of fluke ES products [75], attributing the mitogenic properties of ES products exclusively to the one protein.

GRN protein family members occur in diverse species including bacteria, plants and animals. Human pro-GRN (PGRN) is associated with many aggressive cancers. It is over-expressed in human liver [76, 77], renal [78], breast [79–81], bladder [82] and brain [83] tumours. It may promote cancer progression by stimulating angiogenesis, suppressing anoikis (a form of apoptosis), promotion of tumour invasion and anchorage independence, all of which support tumour expansion in the unfavourable interstitial environment [82, 84, 85]. Preventing the activity of GRN in a range of tumour types, either through gene silencing or antibody neutralization, reduces or entirely inhibits tumour progression [86]. Transfection of fibroblasts with GRN induces serum independent proliferation but does not transform them into

neoplastic cells, suggesting that the protein is probably not oncogenic by itself, but over-expression of PGRN in the SW-13 non-malignant adrenal carcinoma cell line made it highly tumourigenic [87]. Indeed, PGRN is a therapeutic target for liver cancer, particularly HCC. An anti-PGRN monoclonal antibody inhibited tumour growth *in vivo* in nude mice transplanted with human HCC [77]. The anti-PGRN antibody also inhibited growth of hepatoma cells but had little effect on normal liver cells, and inhibited the growth and proliferation of established tumours via the p44/42 MAPK and Akt signalling pathways. These findings demonstrate that GRN is an important factor in the initiation of liver cancer and the migration of cancerous cells. Recent high throughput DNA sequencing has uncovered a multidomain GRN protein from *F. hepatica* and a single domain GRN protein from *C. sinensis* homologous to *Ov*-GRN-1 [72].

Host-parasite interplay and granulin

Why *O. viverrini* secretes such a potent growth factor that acts on host cells is unclear. One potential role for fluke GRN involves wound repair. Mammalian inflammatory cells secrete peptides derived from PGRN [88], and PGRN mRNA is highly induced in dermal fibroblasts and epithelial cells following transcutaneous puncture wounds [89]. Furthermore, human recombinant PGRN increased the accumulation of inflammatory cells, blood vessels and fibroblasts at puncture sites, implying a direct role as a wound-healing growth factor [89]. *O. viverrini* adult worms grasp the bile duct wall with their suckers and feed on the biliary cells, often severely damaging the epithelium. Additional inflammation occurs as a result of the local immune response to resident worms [reviewed in [90]]. *Ov*-GRN-1 might therefore play a role in wound repair at and around the feeding site to minimize the pathology that the parasite causes to the host. Another potential role for *Ov*-GRN-1 is in the “farming” of host cells for nutritional purposes. By promoting growth of cells at the feeding site, the parasite is ensured of a steady supply of nutrients. Blood-feeding leeches secrete a GRN that inhibits thrombin activity [91], and *Ov*-GRN-1 might also perform a similar function to interfere with clot formation while feeding.

Future directions

Progression from opisthorchiasis to CCA is multi-factorial. As emphasized herein, we hypothesize that one of the major predisposing factors towards carcinogenesis is chronic exposure of host tissues to parasite metabolites. To this end, we have begun to catalogue putative carcinogenic molecules from the ES products and surface tegument of *O. viverrini,* taking bioinformatic approaches based on Gene Ontology [71, 72], proteomes [74] and exposure of host cells to parasite products that have been purified using a range of chromatographic techniques (M. Smout and A. Loukas, unpublished). Advances in biotechnology are opening up new fields of research in both parasite and cancer biology alike. Such advances will not only provide answers but will also result in new questions to be addressed. Studies of gene expression changes induced in cells exposed to defined *O. viverrini* proteins will shed light on the molecular mechanisms of disease progression. We and others [92] are using proteomics to search for biomarkers of hepatobiliary damage and/or CCA in cell lines and animal models but ultimately in infected patients. Better diagnostic methods are required to identify individuals at-risk of developing cancer in endemic settings, such that preventative chemotherapy can be specifically targeted at these individuals rather than the currently implemented intermittent mass drug administration programs. Future work on the molecular basis of human-*Opisthorchis* interactions should focus on potential targets for the development of anti-cancer therapies, tailored specifically to fluke-induced CCA. Such approaches will also reveal parasite proteins that might be suitable targets for the development of new drugs as well as recombinant vaccines against opisthorchiasis.

Despite the deployment of mass drug administration programs throughout Thailand, opisthorchiasis is still a major public health problem, and the prevalence of the infection in some areas is increasing [8, 93]. Any advance in elucidating the complex associations of *Opisthorchis* or *Clonorchis* and CCA could assist in reducing both parasite burdens and the incidence of CCA, the most prevalent and fatal of the liver cancers in north-east Thailand [8]. Additionally, the novelty of carcinogenic metazoans may lead to fundamental insights into carcinogenesis in general.

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Figure 1. Images depicting cultural practices that result in liver fluke infection

A. Local creeks in North-East Thailand that harbour fish rife with *O. viverrini* metacercariae. B. Thai Farmer net fishing. C. Infected fish catch. D. Fermented fish that gives consumers the carcinogenic combination of *O. viverrini* and nitrosamines. E. The fermented fish dish, *pla-ra.*

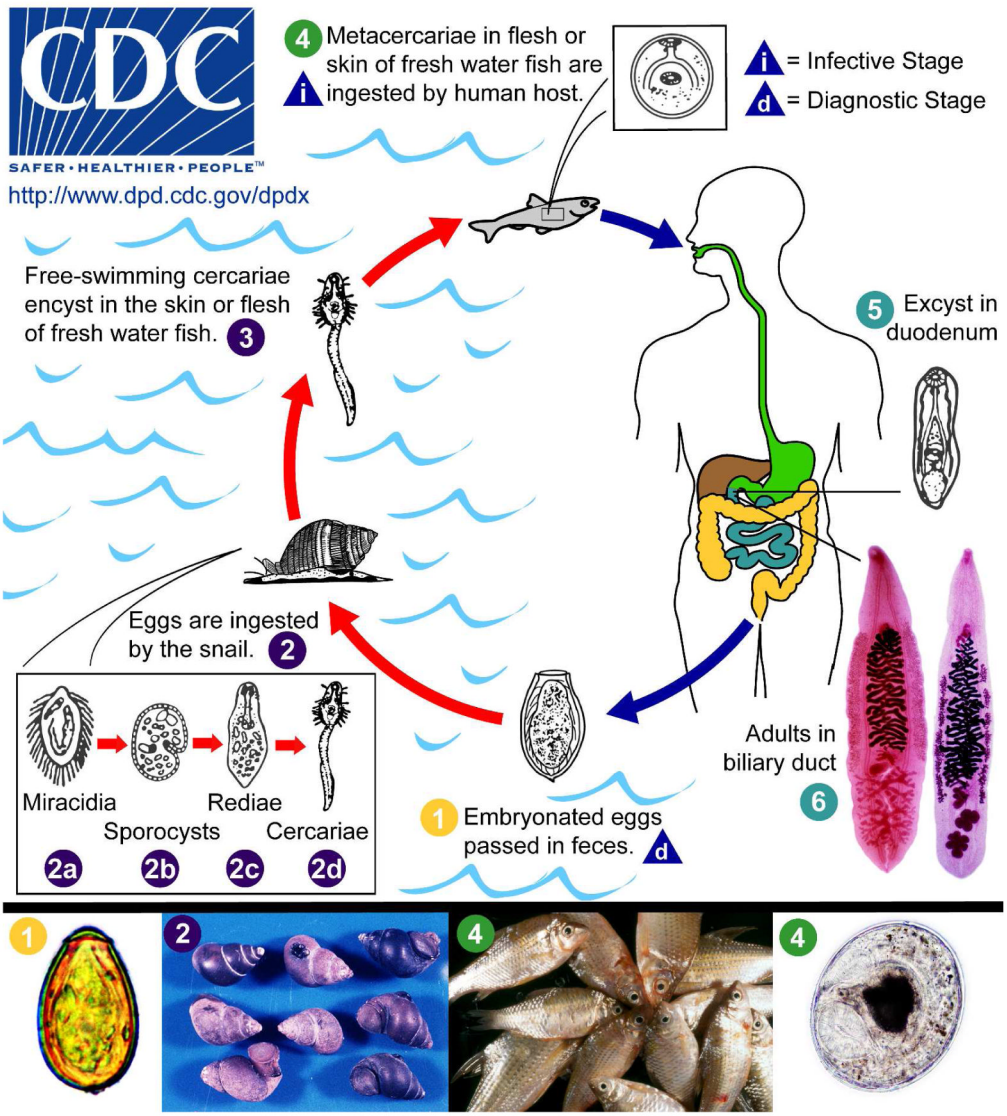

Figure 2. Life Cycles of *O. viverrini* and *C. sinensis.*

Embryonated eggs are discharged in the biliary ducts and in the stools (stage 1). Eggs are ingested by a suitable snail intermediate host (2); there are more than 100 species of snails that can serve as intermediate hosts. Each egg releases a miracidium (2a), which morphs through several developmental stages (sporocyst [2b], redia [2c], to become a cercaria [2d]). Cercariae are released from the snail and after a short period as free-swimming larvae in fresh water, they penetrate the flesh of freshwater fishes, such as *Cyclocheilichthys armatus* or *Puntius leiacanthus*, where they encyst as metacercariae (3). Humans are infected through ingestion of undercooked, salted, pickled, or smoked freshwater fishes (4). After ingestion, the metacercariae excyst in the duodenum (5) and ascend the biliary tract through the Ampulla of Vater. Maturation to adulthood (right, *O. viverrini*; left, *C. sinensis*) takes approximately one month (6). The adult flukes reside in the small and medium-sized bile ducts. In addition to humans, carnivorous animals can serve as reservoir hosts [90].

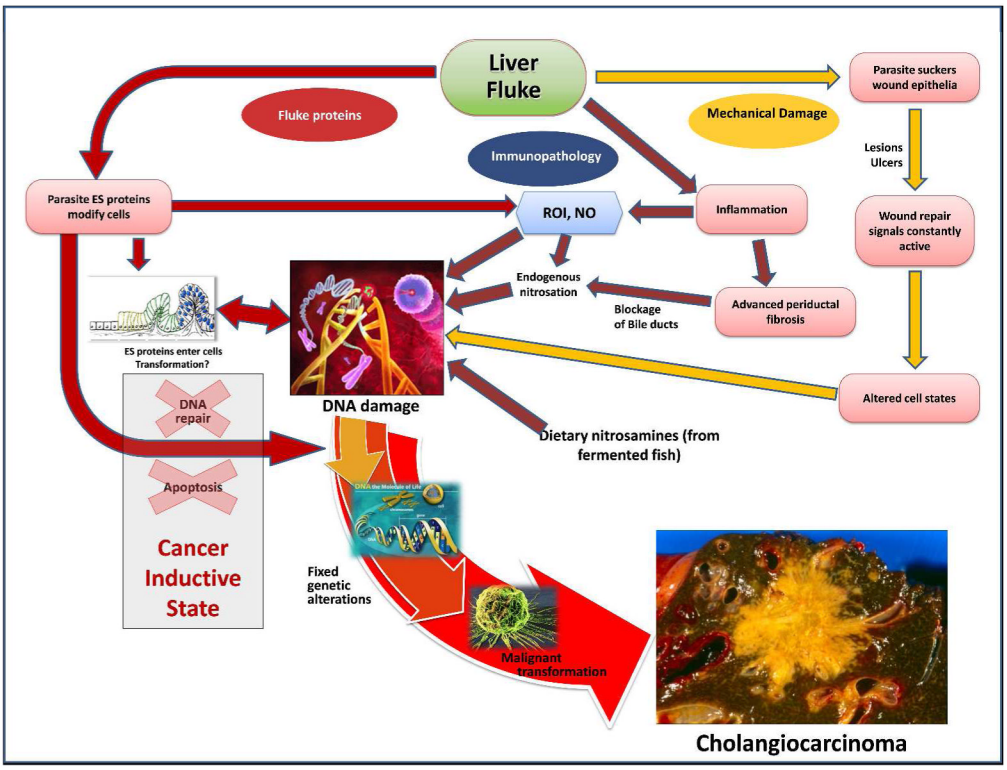

Figure 3. Hypothesized pathways of pathogenesis of opisthorchiasis-associated cholangiocarcinoma

The liver fluke *Opisthorchis viverrini* induces damage to the bile duct tissue via at least three distinct pathways: (1) mechanical damage to biliary epithelia caused by feeding parasites (yellow arrows); (2) inflammation-induced immunopathology, particularly due to reactive oxygen intermediates (ROI) and nitric oxide (NO) (blue arrows); (3) direct effects of fluke secreted proteins on biliary epithelia including cell proliferation induced by parasite-derived growth factors (red arrows). These pathways converge to result in oxidative DNA damage and excessive, chronic cell proliferation. Damaged DNA/genes after successive replications become fixed, leading to malignant transformation of the transformed cholangiocytes. Adapted from [94].

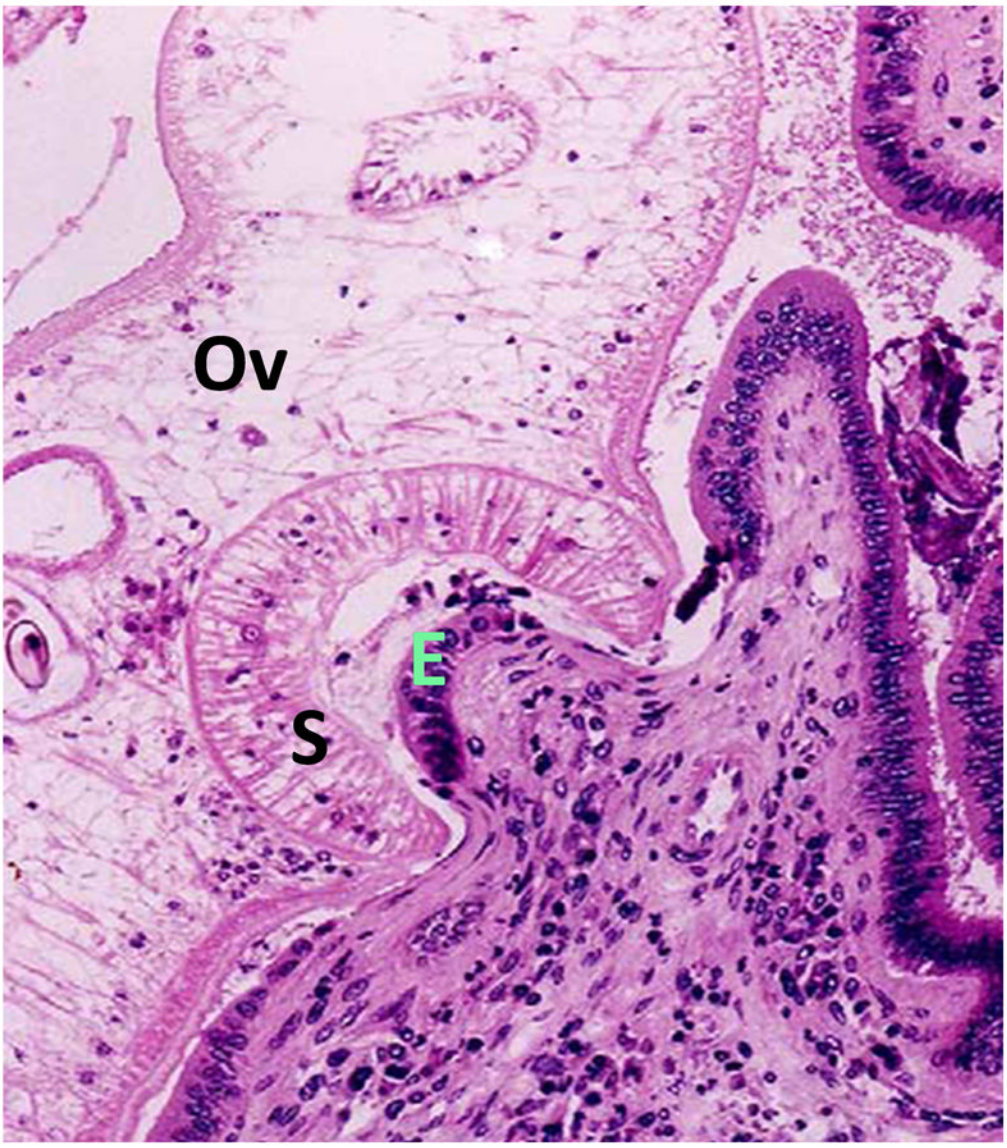

Figure 4. *Opisthorchis* in a liver section

The ventral sucker (S) of the liver fluke (Ov) is attached to the bile duct epithelium (E) [41]. Note the infiltration of eosinophils into the site of inflammation.

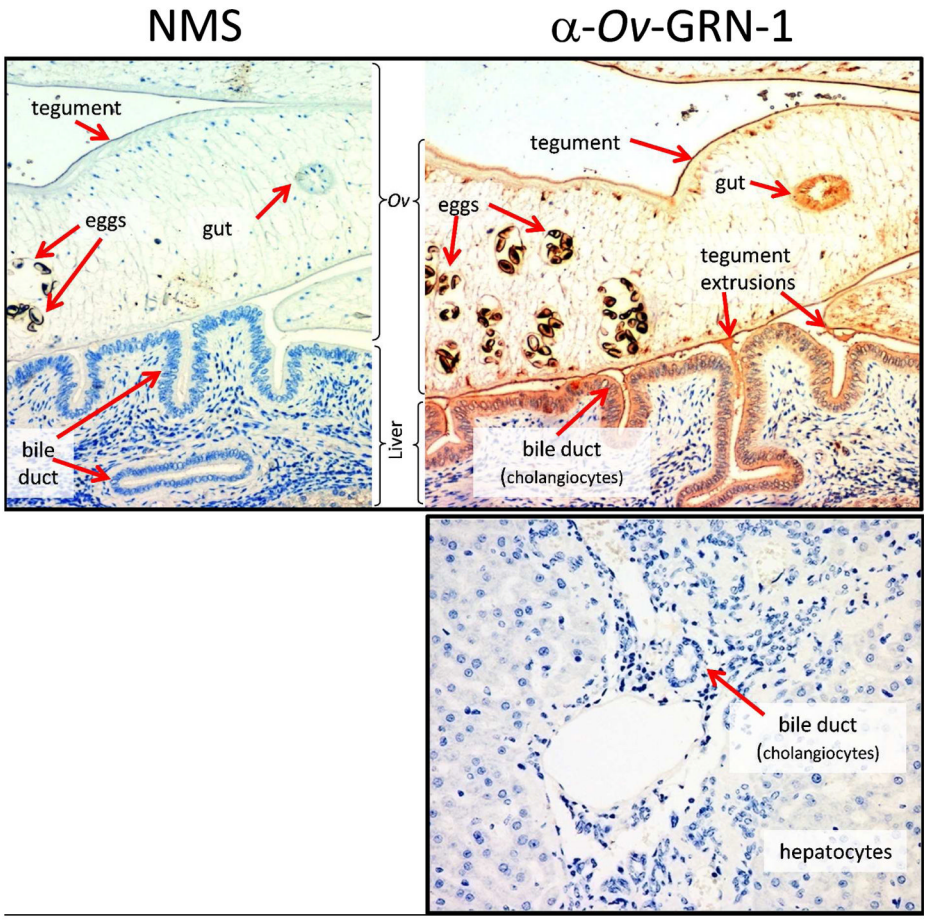

Figure 5. Immunolocalisation of *Ov*-GRN-1 in histological sections of adult *O. viverrini* in the bile ducts of experimentally infected hamsters

The left panel was probed with IgG purified from control normal mouse serum (NMS); the right panel was probed with anti-*Ov*-GRN-1 IgG. The bottom panel shows a liver section from an uninfected hamster that was probed with anti-*Ov*-GRN-1 IgG. All three sections were stained with peroxidase staining revealed as a brown/rust colored deposit and Mayer's Haematoxylin counterstained the nuclei in blue. Red arrows highlight the regions within the *O. viverrini* parasite and bile duct tissue that stained positive for *Ov*-GRN-1 [75].

Table 1

Malignancies Attributable to Infectious Agents [2, 90]

Pathogen Type	Pathogen	Malignancies	Non-Malignant Disease	Percent of annual cancers
Viruses	Human papilloma virus: 13 carcinogenic strains (HPV)	cervix, vulva, vagina, penis, anus, oral cavity, oropharynx and tonsil	Warts, condyloma	**5.2**
	Hepatitis B or C viruses (HBV and HCV)	Liver: Hepatocellular carcinoma (HCC), non-HLP (Hepatitis C only)	Hepatitis, cirrhosis	**4.9**
	Epstein Barr virus (EBV)	Nasopharyngeal, extranodal NK/T-cell LP (nasal type), HLP, Burkitt's LP, immune-suppression-related non-HLP	Infectious mononucleosis	**1**
	Human immunodeficiency virus, type 1 (HIV-1)	Kaposi's sarcoma, non-HLP, HLP, cervix, anus, conjunctiva	AIDS	**0.9**
	Human T-cell lymphotrophic virus type 1 (HTLV-1)	Adult T-cell leukaemia and LP		**0.03**
Bacteria	*H. pylori*	Non-cardia gastric carcinoma, MALT gastric LP	Gastritis, peptic ulcers	**5.5**
Helminths	*S. haematobium* (blood fluke)	Urinary bladder cancer	Schistosomosis	**0.1**
	O. viverrini and C. sinensis (liver flukes)	Liver: Cholangiocarcinoma (CCA)	Opisthorchiasis and Clonorchiasis	**0.02**
			Total	**17.80%**

HLP: Hodgkin's lymphoma, LP: Lymphoma, MALT: Low-grade B-cell mucosa-associated lymphoid tissue