Infection with \textit{Opisthorchis felineus} induces intraepithelial neoplasia of the biliary tract in a rodent model

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Abstract

The liver fluke \textit{Opisthorchis felineus} is a member of the triad of epidemiologically relevant species of the trematode family \textit{Opisthorchiidae}, and the causative agent of opisthorchiasis felinea over an extensive range that spans regions of Eurasia. The International Agency for Research on Cancer classifies the infection with the liver fluke \textit{Opisthorchis viverrini} and \textit{Clonorchis sinensis} as group 1 agents and a major risk factor for cholangiocarcinoma. However, the carcinogenic potential of the infection with \textit{O. felineus} is less clear. Here, we present findings that support the inclusion of \textit{O. felineus} in the Group 1 list of biological carcinogens. Two discrete lines of evidence support the notion that infection with this liver fluke is carcinogenic. First, novel oxysterol-like metabolites detected by liquid chromatography-mass spectroscopy in the egg and adult developmental stages of \textit{O. felineus}, and in bile, sera, and urine of liver fluke-infected hamsters exhibited marked similarity to oxysterol-like molecules known from \textit{O. viverrini}. Numerous oxysterols and related DNA-adducts detected in the liver fluke eggs and in bile from infected hamsters suggested that infection-associated oxysterols induced chromosomal lesions in host cells. Second, histological analysis of liver sections from hamsters infected with \textit{O. felineus} reveals a significant increase in intraepithelial neoplasia of the biliary tract.
felineus confirmed portal area enlargement, inflammation with severe periductal fibrosis and changes in the epithelium of the biliary tract characterized as biliary intraepithelial neoplasia, BilIN. The consonance of these biochemical and histopathological changes revealed that O. felineus infection in this rodent model induced precancerous lesions conducive to malignancy.

### Abbreviations

<table>
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<th>Abbreviation</th>
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<tr>
<td>BilIN</td>
<td>biliary intraepithelial neoplasia</td>
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<td>CCA</td>
<td>cholangiocarcinoma</td>
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<td>LC-MS</td>
<td>liquid chromatography-mass spectroscopy</td>
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### Introduction

The liver fluke *Opisthorchis felineus* is one of the causative agents of opisthorchiasis. Formerly, *O. felineus* occurred primarily within the territory of the Russian Federation, especially in Western Siberia, the Ukraine, Byelorussia, Kazakhstan, and the Baltic countries (1). However, it is now increasingly seen in other European regions, including Italy where outbreaks of acute human infection have been reported recently (2–5). Worldwide, infection with *O. felineus* is responsible for about one in 10 cases of opisthorchiasis (~1.6 million out of 17 million cases (6)). This food-borne liver fluke is a member of the trematode family Opisthorchiidae, which also includes the epidemiologically and clinically relevant species, *Opisthorchis viverini* and *Clonorchis sinensis*. The International Agency for Research on Cancer (IARC) classifies the infection with *O. viverini* and *C. sinensis* as group 1 carcinogens (7), definitive risk factors for cholangiocarcinoma (CCA) (8,9). The clinical manifestations and pathology induced by chronic infection with all of these opisthorchid flukes are similar (10,11). However, the carcinogenic potential, physiology, molecular biology and mechanisms of host–parasite interaction are less well studied for *O. felineus* than *O. viverini* and *C. sinensis*. There remains insufficient awareness of liver fluke infection with *O. felineus* as a problem for public health; nonetheless, infection with this species impact millions of people with severe morbidity and the geographical range of opisthorchiasis felines continues to expand and to emerge in new locations (12).

*Opisthorchis felineus* has a complex life cycle involving three hosts; a gastropod snail and a ctenophore fish serve as first and second intermediate hosts, respectively, and a mammalian piscivorous definitive host. Infection of the definitive host follows the consumption of fish contaminated with metacercariae. Bears, cats, dogs, foxes and people are all permissive definitive hosts where the parasite develops into adults within the intra- and extra-hepatic bile ducts and the gallbladder. Human infection is especially routine where consumption of smoked or uncooked fish is a dietary preference (5,13–15). The metacercaria excysts in the duodenum and the juvenile parasite ascends through the ampulla of Vater into the bile ducts, where the adult worm develops in 4–6 weeks. This liver fluke is a hermaphrodite, long-lived and dwells within the biliary tract, feeding on epithelial cells, host blood and bile contents (11).

Opisthorchiasis *felineus* induces cholecystitis, cholangitis, gallbladder dysfunction, and hepatic abscess. Pathological changes that follow infection include chronic, proliferative cholangitis and pancreatic canaliculitis accompanied by tissue fibrosis (10,11). Available data indicate that the prevalence of liver cancer, largely diagnosed as CCA (16–18), is three times higher in liver fluke endemic regions of Western Siberia than in Russia at large (6). To date, there is a modest catalogue of supporting information for a role of infection with *O. felineus* as a risk for CCA (3,13,15), although this aspect has not been received sufficient investigation (6).

This epidemiological situation supports an association of infection-associated cancer related with chemical carcinogenesis along the lines of the pioneering report of Miller and Miller (19). To further investigate this phenomenon, here we undertook both biochemical and histopathological investigations in hamsters experimentally infected with *O. felineus*, a widely studied model of human opisthorchiasis and liver fluke infection-induced hepatobiliary disease including CCA (8,10,11). We characterized novel oxysterol-like metabolites using liquid chromatography-mass spectroscopy (LC-MS) in the adult and egg stages of the liver fluke and in bile, sera and urine of infected hamsters. The presence of diverse forms of DNA-adducts suggested that infection-associated oxysterols might be responsible for chromosomal lesions in host cells. In parallel, histopathological analysis revealed inflammation, severe periductal fibrosis and changes in the epithelium of biliary ducts identified as biliary intraepithelial neoplasia (BilIN), an established precancerous lesion that precedes intrahepatic CCA (20,21).

### Materials and methods

#### Ethical statement

Procedures undertaken complied with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm. Syrian hamsters (Mesocricetus auratus) were purchased from the stock of the Puschino Animal Facility (Russia) and bred at the Animal Facility of the ICG SB RAS (RFMEFI61914X0005) (Russia). The hamsters were maintained according to protocols approved by the Committee on the Ethics of Animal Experiments of the Institute of Cytology and Genetics (Permit Number: 25 of 12.12.2014).

#### Parasites, hamsters and experimental design

Metacercariae of *O. felineus* were collected from naturally infected Leuciscus idus from the Ob River, Novosibirsk city, Western Siberia, and isolated from fish muscle tissues digested with pepsin–HCl overnight at 37°C. Territories where collection of the fish was undertaken were neither conservation areas nor private property, nor otherwise protected; hence, fishing permits were not required. Leuciscus idus is not considered endangered or rare, and the fishing methods complied with the Federal Law N166-F3 of 20.12.2004 (ed. 18.07.2011) ‘Fishing and conservation of water bio-resources’.

Male Syrian golden hamsters aged 6–8 weeks were orally infected with 50 *O. felineus* metacercariae. The rodents were housed at three or four per cage under conventional conditions and received a stock diet and water ad libitum. Control non-infected hamsters (*n* = 4) and *O. felineus*-infected hamsters (*n* = 4) were necropsied 12 weeks after infection.

#### Sample collection and pathological studies

Hamsters were euthanized using carbon dioxide after which blood was collected by cardiac puncture. Blood was centrifuged at 3000g for 10 min at 4°C to obtain the serum. Ascorbic acid was added to 1 mg/ml (22–24) and the sera aliquoted and stored at −80°C. Urine was aspirated from the urinary bladder by syringe; ascorbic acid was added to 1 mg/ml. Urine samples were aliquoted and stored at −80°C. Bile was collected from the gallbladder by syringe aspiration; ascorbic acid was added to 1 mg/ml, aliquoted, and stored at −80°C.

Liver flukes recovered from livers of hamsters were thoroughly washed with sterile saline (0.9% NaCl) and incubated at 37°C, 5% CO₂ for...
24 h in RPMI 1640 culture medium (Life Technologies) supplemented with 1% antibiotic/antimycotic (Sigma–Aldrich) and 1% glucose. Eggs laid in vitro from these cultured adult worms were collected by centrifugation of the culture medium, thoroughly washed with PBS and stored at −80°C. Soluble extracts of the flukes were prepared by sonication (5 × 5 s bursts, output cycle 4, Vibra Cell, Sonics) in PBS supplemented with protease inhibitors [500 μM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 0.3 μM aprotinin, 10 μM PMSF, 10 μM bestatin and 10 μM leupeptin] (M221, Amresco), followed by 30-min centrifugation at 10,000 rpm and 4°C. The supernatant was collected and protein concentration determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Ascorbic acid was added to 1 mg/ml to the extracts; aliquots were stored at −80°C.

The livers were dissected and immersed in 10% buffered formalin (Biovitrum, Russia). After fixation overnight at 4°C, specimens were dehydrated through a graded series of ethanol to 100% ethanol, cleared in xylene, and soaked in melted paraffin. Thereafter, specimens were embedded in paraffin using Microm (Microm, UK) and 4-μm-thick sections were prepared using a microtome. For histopathological analysis, tissue sections were stained with haematoxylin and eosin (H&E) (11). The slides were examined under a light microscope (Axioskop 2 Plus; Zeiss, Germany), with magnification ×40, ×100 and ×400, and micrographs captured using a Nikon DS-5M-Li camera.

**Histopathology and Immunohistochemistry**

Four 4-μm-thick paraffin-embedded sections were prepared as above from both infected and non-infected hamsters for histopathological and immunohistochemistry analysis. Slides were stained with H&E and Masson’s trichrome. For immunohistochemistry, formalin-fixed, paraffin-embedded tissue slides were screened for accumulation of p53 and proliferation (Ki-67) by using the streptavidin/biotin peroxidase and mouse monoclonal antibodies. The p53 protein was determined with clone DO-7 (DO-7, Dako, Glostrup, Denmark), Ki-67 with clone MIB-1 (MIB1, 1:100; Dako). Briefly, slides were deparaffinised with xylene, rehydrated through a graded ethanol series, micro waved for 15 min in boiling citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0), and exposed to 3% hydrogen peroxide in methanol for 20 min. After blocking the endogenous peroxidase, slides were incubated in protein block solution (DakoCytoMation). Thereafter, the samples were incubated overnight at 4°C with each of the primary antibodies and treated with secondary antibodies conjugated to horseradish peroxidase (HRP; Dako) or alkaline phosphatase (Vector Laboratories). The secondary antibodies were developed in 3,3’-diaminobenzidine tetrahydrochloride as the substrate, after which slides were lightly counterstained with hematoxylin. Negative control sections were probed with the criteria of the International Observer Agreement Guidelines for BilN (20,21).

**Sample preparation and LC-MS/MS**

Samples were prepared and processed for LC diode array detection electron spray ionization mass spectrometry as described (25,26). Since murine displays an acceptable chromatographic performance in terms of separation and sensitivity, with short gradient times (27), this solvent was added to 20% by volume of the samples of serum, urine and bile from control and infected hamsters, and to extracts of *O. felineus* adult worms and of liver fluke eggs. Subsequently, bile samples were centrifuged (MiniStar silverline VWR, at 4000 rpm for 10 min after which the supernatant was collected; 25 μl of each sample was injected into LC-MS/MS. Duplicate samples were analyzed.

Higher performance liquid chromatography coupled with mass spectrometer (MS) was employed to investigate metabolite species from the hamsters and liver flukes. The MS analysis was performed within a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), fitted with a ultraviolet (UV) photo diode array (PDA) detector. Analysis involved a single nucleosil C18-column (250 mm × 4 mm internal diameter, 5 μm particle diameter, end-capped), proceeding at a flow rate of 0.3 ml/min. Eluates were monitored for 75 min, run with a mobile phase gradient of 0–5 min, 100% A; 5–10 min, linear gradient from 100% to 80% A; 10–15 min 80% A; 15–50 min, linear gradient from 80% to 40% A; 50–65 min, 40% A, 65–75 min, linear gradient from 40% to 100% B. Washing the column for 15 min with acetonitrile between each sample was undertaken in order to minimize carry-over and to stabilize the column. Data were collected in negative electrospray ionization negative mode scanning a mass to charge ratio (m/z) range of 50–2000. The capillary voltage of the electrospray ionization was 28 kW, capillary temperature was 310°C, flow rates of the sheath gas and auxiliary N₂ were set to 40 and 10 (arbitrary unit as provided by the software settings), respectively, and gas temperature was 275°C. At the outset, the workflow was undertaken using two samples for each biofluid, for example, sera from two groups: sera from control hamster followed by sera from infected hamsters, etc. The analysis of developmental stages of *O. felineus* were performed separately from biofluids in order to avoid possible contamination.

**Results**

**Oxysterol-like molecules in developmental stages of *O. felineus***

Using LC-MS/MS, numerous oxysterol-like metabolites were detected in extracts of the adult and the egg stages *O. felineus* (Supplementary Figures S1 and S2 are available at Carcinogenesis Online). These extracts contained not only numerous novel and specific oxysterol-like metabolites, for example m/z 321 (m/z = mass to charge ratio) but also DNA-adducts (m/z 698) not detected in controls (Figure 1). The oxysterol-like metabolites of *O. felineus* were similar to those described for *O. viverrini*, for example m/z 385 and 438 (25), The proposed structures displayed ramification at C-17, that is discrete and variable chains linked to carbon 17 of the steroid ring, in similar fashion to bile acids and their conjugated salts (Supplementary Figure S2 is available at Carcinogenesis Online). Supplementary Figures S1 and S2, available at Carcinogenesis Online present predicted structures for the m/z species detected in *O. felineus* worms and eggs. These included bile acids, which constitute a large family of steroids carrying a carbonyl group on a side chain (m/z 425) (28), bile alcohols (m/z 362), which have similar products in bile acid biosynthesis or as final products, or free bile acids conjugated in some species, as aldehydes (m/z 340, 356), glycinine (m/z 425, 439) and sulfates (m/z 501, 557). This catalogue of oxysterols included molecular species compatible with the presence of derivatives of catechol estrogens and other components hydroxylated at the steroid ring, including at both C-2 and C-3 and respective oxidized 2,3-quinone (e.g. m/z 326, 461, 471). We speculate that effects of the individual oxysterol-like species on the pathophysiology during infection might be structure-dependent, with metabolic conversions resulting in a mixture of biologically active or inactive forms (25).

**Parasite oxysterol-like molecules in biological fluids during infection**

The chromatographic profiles of sera, urine and bile identified in controls and *O. felineus*-infected hamsters were compared and contrasted with extracts of the parasite (Figure 1 and Supplementary Figure S3, available at Carcinogenesis Online). The presence of oxysterol-related metabolites of parasite origin was unequivocally apparent. Figure 1 and Supplementary Figure S4, available at Carcinogenesis Online summarize the data and highlight similarities in metabolite species present both in *O. felineus* and the infected hamsters. Supplementary Figure S3, available at Carcinogenesis Online presents representative photo diode array and mass spectra chromatograms; samples were analyzed in duplicate and results presented as the average of the m/z
obtained (Supplementary Table S1 is available at Carcinogenesis Online). As noted, a number of the metabolites were seen in both hamster biofluids and the liver flukes (Figure 1). The presence of specific DNA-adducts, for example m/z 617 in bile and m/z 588 in sera (Supplementary Figures S5 and S6, respectively) are available at Carcinogenesis Online, suggested interactions of oxysterol-like metabolites and host cell chromosomal DNA. In addition, other parasite-derived metabolites, for example m/z 430 in bile and m/z 539 in serum (Supplementary Figures S5 and S6 are available at Carcinogenesis Online) might interact farther with host DNA. However, similar shared metabolites were not detected in urine of infected hamsters (Figure 1 and Supplementary Figure S7, available at Carcinogenesis Online).

**BilIN associated with infection with O. felineus—BilIN 1/2/3**

Sections of the livers of hamsters infected for 12 weeks revealed that the bile ducts were lined by cells with enlarged nuclei, exhibiting pseudo-stratification, hyperchromatism, loss of polarity, nuclear crowding, and mitotic metabolites with low-grade and intermediated-grade dysplasia (Figures 2–5). First, control, the epithelium of non-infected hamsters displayed a cuboidal to low-columnar phenotype with amphiphilic cytoplasm (Figure 2). By contrast, during infection, bile ducts were lined by epithelium with enlarged nuclei, pseudo-stratification, hyperchromatism, atypical hyperplasia and papillary duct lesion with atypia, loss of polarity, nuclear crowding and mitotic figures, low-grade dysplasia and some foci of moderate dysplasia, as described for BilIN (20,21). A transition from normal duct epithelium to an atypical lesion should suggest cancerization of the duct or ductile (Figure 2, panels B, B′ and C). The major histological changes in the gallbladder and extrahepatic bile ducts comprised adenomatous hyperplasia, epithelial hyperplasia and chronic inflammation. Masson’s trichrome stains type I collagen a greenish/bluish hue, and is widely used to assess the degree of fibrosis. Severe periductal fibrosis was evident (Figure 3). Granulomatous inflammation, mononuclear cell and eosinophil infiltration in the portal area in response to entrapped parasite eggs in the periductal tissue and liver parenchyma also were evident (Figure 4A and B). These morphological alterations and phenotypic profile foci of BilIN were microscopically located in the intrahepatic large bile ducts. Thus, we observed BilIN-1 and 2, but also possible BilIN-3. However, BilIN-3, which occasionally shows p53 expression, was not readily evident. Additionally, bile duct epithelia showed high proliferative index (Ki-67) translatating intraepithelial neoplasia (Figure 5A). Expression of p53 was not evident in the bile duct epithelium of infected hamsters (Figure 5B).

**Discussion**

Host–parasite interactions contribute to the molecular mechanisms of opisthorchiasis-associated pathology and clinical complications, including cholelithiasis and cholangiocarcinogenesis (8). Specific liver fluke-derived metabolites might directly damage the nuclear DNA of cholangiocytes, leading to malignant transformation. The introduction of enhanced LC-MS/MS approaches that require only minimal sample sizes and facile protocols has improved the analysis of conjugated bile acids (29). Previously, we developed an LC-MS/MS method to identify steroid-based molecules in extracts of O. viverrini and Schistosoma haematobium and biofluids from human cases of urogenital schistosomiasis, involved in the estrogen metabolism (25,26,30). Using similar approaches here, we identified oxysterol-like metabolites from the egg and adult developmental stages of O. felineus with m/z, mass to charge ratio identical to those of O. viverrini. Moreover,
the analysis of sera, bile and urine of hamsters infected with O. felineus, and comparisons with chromatograms from adult and egg developmental stages, revealed metabolites with the same or similar m/z, for example metabolites with m/z 325 for sera and 339 for bile.

Similar to O. viverrini, analysis of O. felineus revealed the presence of oxysterols of diverse forms: bile alcohols, bile aldehydes and reconjugated as glycine or sulfates. Catechol forms (e.g. m/z 461) also were identified (Supplementary Figure S1 is available at Carcinogenesis Online). Oxysterols observed in the liver fluke O. felineus might, at least in part, arise from nonenzymatic reaction with oxidative free radical-like oxygen and nitrogen species. It is also feasible that they could originate enzymatically as products of cytochrome P450 family enzymes. Indeed, a sole member of the cytochrome P450 family of genes is known from the genome of O. felineus (31,32). Chronic inflammatory responses to O. viverrini generate reactive oxygen species and reactive nitrogen species (33), which might react with biomolecules within the inflammatory tissue such as the bile ducts during opisthorchiasis felinea. Biliary tract epithelia and surrounding tissue of hamsters infected with O. felineus displayed forms of BilIN. BilIN has been defined as a precursor lesion of invasive adenocarcinoma in the biliary tract (20,21), and represents the multistep process in carcinogenesis. BilIN represents a spectrum of proliferative and/or cytological atypical lesions of the large intrahepatic bile ducts, considered a major transformation within the pathway leading to intrahepatic CCA. BilIN lesions are characterized by a flat or micropapillary dysplastic epithelium in the bile ducts and, according to international guidelines, have been used

Figure 2. Biliary histological features observed in the liver biopsies from control, non-infected hamsters. (A) Normal portal unit with bile duct, hepatic arteriole, portal venule, and a clearly defined limiting plate (magnification ×200). The smaller or interlobular bile ducts are lined by cuboidal or low columnar epithelium. No evidence of inflammation (H&E staining). (A1) defines magnified area (magnification ×400) of normal portal unit. Biliary histological features observed in the liver biopsies of hamsters infected with Opisthorchis felineus. (B, B’ and C) Biliary obstruction caused by the O. felineus worm with portal area enlargement (H&E staining) (magnification ×100). (B1) Dashed line defines magnified area (magnification ×100). (A) dashed lines define magnified areas as (B’1) (magnification ×400) and (C) demonstrated the biliary obstruction caused by O. felineus with portal area enlargement. Bile ducts were lined by enlarged nuclei, with pseudo-stratification, hyperchromatism and some loss of polarity, nuclear crowding, mitotic figures (C1, C’1—arrows) and low-to moderate-grade of dysplasia. Evidence of flat or micropapillary dysplastic epithelium in the bile duct; these lesions are referred as biliary intraepithelial neoplasia (BilIN). (C) Epithelium lining a large intrahepatic bile duct displays flat hyperplasia with dysplastic changes (BilIN1/2). (C1) (magnification ×400), Note increased cellularity, modestly increased pseudo-stratification, and nuclear irregularities including variation in size and polarity, cytologic atypia including presence of nucleoli and loss of polarity (BilIN2).
instead of traditional biliary dysplasia. These pre-malignant lesions are classified into three grades: BilIN-1 (low-grade lesion), BilIN-2 (intermediate-grade lesion) and BilIN-3 (high-grade lesion). Here, BilIN-1 and BilIN-2 lesions were characterized unequivocally in the *O. felineus*-infected hamsters. The findings strongly suggested that infection with *O. felineus* is associated with intracellular mechanisms that eventually trigger neoplastic transformation of cholangiocytes, and promote biliary carcinogenesis. Multifocal BilIN were clear histopathological features observed in this rodent model. These findings support earlier observations that *O. felineus* might induce CCA, at least in hamsters (11). It is reasonable to speculate that the pathobiology of *O. felineus* and *O. viverrini* infections are similar (15). Both species of *Opisthorchis* injure the host through direct physical and/or immunopathological processes and indeed damage from infection with *O. felineus* is even more marked than *O. viverrini* (10).

Atypical hyperplasia and dysplasia of the epithelium of the bile duct and egg granulomas in the periductal tissues and liver parenchyma are potential precancerous lesions. Eggs may extravagate into the adjacent parenchyma and incite a granulomatous response and infiltration of lymphocytes, plasma cells and eosinophils, and fibrosis. We hypothesize that parasite-specific oxysterols contribute to the granuloma formation and progression, given the broad range of these metabolites identified in the eggs. The hypothesis that the eggs provoke granulomatous inflammation also is supported the findings in bladder cancer lesions induced by urogenital schistosomiasis where schistosome eggs at the nidus of the granulomatous lesions generate estrogen-like molecules that damage the DNA of urothelial cells.
Inflammatory responses against schistosome eggs in the wall of the urinary bladder represent early events in the pathogenesis of urogenital schistosomiasis (34).

We posit that carcinogenesis associated with chronic infection with *O. felineus* mimics malignant transformation induced by infection with *O. viverrini* and *C. sinensis*. The findings presented here support this hypothesis. The findings revealed, first, the presence of cholesterol-derived metabolites of liver fluke origin. Second, they revealed that these oxysterol-like metabolites exhibited striking similarity to those of *O. viverrini*. Third, they revealed unequivocal signals of BilIN-1 and BilIN-2. Oxysterols are products of oxidation of cholesterol that arise through enzymatic (P450) or non-enzymatic processes. Oxysterols display mutagenic, genotoxic, pro-oxidative and pro-inflammatory properties that can contribute to malignancy. Associations between oxysterols and the development and progression of cancer of colon, lung, breast and bile ducts have been proposed (35, 36). As with infection with *O. viverrini* and also during urogenital schistosomiasis (8, 37), we speculate that *O. felineus* produces oxysterols excreted to the biliary system where they may cause lesions in chromosomal DNA of the cholangiocytes lining the biliary tree, which result in due course in BilIN.

It has been generally accepted that CCA tumors in this hamster model occur only as a result of a combined action of infection with the liver flukes, *O. felineus*, *O. viverrini* and *C. sinensis* and exposure to *N*-nitrosodimethylamine (11, 38, 39). However, the nature and occurrence of precancerous lesions during liver fluke infection has remained poorly understood. Here, we assessed the expression of Ki67 and p53, and presence of intraepithelial bile duct neoplasia and severe fibrosis. Together these data indicate that precancerous changes occur in hamsters without exposure to an exogenous carcinogen such as *N*-nitrosodimethylamine. Concerning how CCA develops, the role of liver flukes has been assigned as a tumor cell growth promoter, whereas the role of *N*-nitrosodimethylamine is a mutagenic cancer inducer. By contrast, we now demonstrate the presence of parasite-specific oxysterol metabolites conjugated with DNA bases presumably derived from host tissues. The presence of these adducts provides cogent indirect evidence of both mutagenic and carcinogenic potential of infection with *O. felineus*. Thus, the role for liver flukes is not restricted only to promotion of chronic inflammation, establishment of conditions favorable to the promotion and proliferation of incipient cancer cells, and tumor growth, but now can be seen to also include mutagenesis capable of initiation of the malignant transformation. This represents the key and notable advance provided by these new findings.

To date, the International Agency for Research on Cancer categorizes infection with *O. felineus* as a group 3 carcinogen, i.e. there is insufficient evidence yet for its classification as carcinogenic, unlike the situation with the closely related liver flukes *O. viverrini* and *C. sinensis*, and the blood fluke *S. haematobium* which are group 1 carcinogens—definitely carcinogenic in humans. However, our findings highlight the need for reconsideration of this classification with respect to infection with *O. felineus* (7). To summarize and conclude, we report that *O. felineus* flukes secreted/excreted oxysterol-like molecules into the bile, which in turn may react with host chromosomal DNA to form apurinic sites causing error-prone base excision repair leading to mutations and, ultimately, initiate carcinogenesis (25, 28, 40). We also document clearly that infection with *O. felineus* induces BilIN. The consonance of findings that demonstrate both the presence of these metabolites and of BilIN-1 and BilIN-2 indicates that *O. felineus* infection induces neoplastic transformation of cholangiocytes. Hence, this infection can be expected to promote growth.

Figure 5. Immunohistochemistry to reveal expression of Ki-67 and p53 in liver from hamsters infected with the liver fluke *Opisthorchis felineus* (A) (magnification ×100), epithelium of bile ducts with high proliferative index (Ki-67) translating intraepithelial neoplasia (A1, magnification ×400x). (B and B1, magnification ×100), expression of p53 was not observed in non-neoplastic epithelium of the bile ducts, as well as in BilIN-1 and BilIN-2 (B1). Eggs of *O. felineus* (arrow).
of biliary cancers. Deeper investigation is warranted in order to decipher relationships between liver fluke oxysterols and malignancy, including during opisthorchiasis felinea in humans.

**Supplementary Material**

Supplementary data are available at Carcinogenesis online.

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