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Proteomic characterization of the internalization of *Opisthorchis viverrini* excretory/secretory products in human cells

Sujittra Chaiyadet [a,b], Michael Smout [b], Thewarach Laha [c], Banchob Sripa [d], Alex Loukas [b], Javier Sotillo [b,*]

[a] Biomedical Sciences, Graduate School, Khon Kaen University, Khon Kaen, Thailand
[b] Centre for Biodiscovery and Molecular Development of Therapeutics, Australian Institute of Tropical Health and Medicine, James Cook University, Cairns, Queensland, Australia
[c] Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
[d] Department of Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand

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ABSTRACT

The association between liver fluke infection caused by *Opisthorchis viverrini* and cholangiocarcinoma (CCA — hepatic cancer of the bile duct epithelium) has been well established. Multiple mechanisms play a role in the development of CCA, but the excretory/secretory products released by *O. viverrini* (*Ov*ES) represent the major interface between the parasite and its host, and their uptake by biliary epithelial cells has been suggested to be responsible for proliferation of cholangiocytes, the cells that line the biliary epithelium. Despite recent progress in the study of the molecular basis of *O. viverrini*–host interactions, little is known about the effects that *Ov*ES induces upon internalization by host cells. In the present study we incubated non-cancerous human cholangiocytes (H69) and human colon cancer (CaCo-2) cells with *Ov*ES and performed a time-course quantitative proteomic analysis on the cells to determine the early changes induced by the parasite. Different KEGG pathways were altered in H69 cells compared to Caco-2 cells: glycolysis/gluconeogenesis and protein processing in the endoplasmic reticulum. In addition, the Reactome pathway analysis showed a predominance of proteins involved in cellular pathways related to apoptosis and apoptotic execution phase in H69 cells after incubation with *Ov*ES. The present study provides the first proteomic analysis to address the molecular mechanisms by which *Ov*ES products interact with host cells, and Sheds light on the cellular processes involved in *O. viverrini*-induced CCA.

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1. Introduction

The parasitic platyhelminth *Opisthorchis viverrini* represents a major public health problem in different countries of Southeast Asia (Lao PDR, Cambodia, southern Vietnam and Thailand), where approximately 10 million people are infected. This liver fluke has a complex life cycle that involves snails and freshwater cyprinoid fish as intermediate hosts, and piscivorous mammals (including humans, cats and dogs) as definitive hosts [1]. The infection is acquired through the ingestion of raw or undercooked fish containing the infective stage (metacercariae), which excysts in the duodenum allowing the juvenile worms to migrate to the bile ducts where they mature and feed on the biliary epithelium. The clinical complications associated with *O. viverrini* infection include cholangitis, obstructive jaundice, hepatomegaly, periductal fibrosis, cholecystitis and cholelithiasis [2,3]; however, the major problem associated with opisthorchiasis is cholangiocarcinoma (CCA), a fatal bile duct cancer [4–6]. Incidence rates of CCA range from 93.8 to 317.6 per 100,000 people/year in some districts of Northeast Thailand and prognosis is poor [4,7].

Despite recent advances in our understanding of the molecular basis of *O. viverrini*–host interactions, the mechanisms by which this liver fluke promotes CCA are not well understood. The pathogenesis of opisthorchiasis is likely multifactorial and includes (i) mechanical irritation to biliary epithelia caused by the feeding activities of the parasite, (ii) prolonged infection-related inflammation and (iii) the immunopathology induced by the excretory/secretory (ES) products released by the parasite [1,6,8,9]. In fact, *O. viverrini* ES products can be internalized by host biliary cells and are highly immunogenic and mitogenic to the biliary epithelium [3]. A proteomic study identified more than 300 proteins present in the ES products and tegument of *O. viverrini* [10], including homologues of human growth factors that promote host cell proliferation [11,12]. Sripa & Kaewkes [13] showed that the internalization of *O. viverrini* ES products by human cholangiocytes was associated with heavy inflammatory cell infiltration; and more recently Chaiyadet et al. [14] showed that the clathrin-mediated endocytosis pathway was implicated in the internalization of these ES products by cholangiocytes in vitro. In this sense, TLR-4 was suggested to play a key role since TLR-4 mRNA expression is induced by *O. viverrini* ES products in a human cholangiocyte cell line via an LPS-independent mechanism [15].

* Corresponding author at: Centre for Biodiscovery and Molecular Development of Therapeutics, James Cook University, Cairns 4878, Queensland, Australia.
E-mail address: javier.sotillo@jcu.edu.au (J. Sotillo).

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Despite these recent advances, the cellular changes at a proteomic and genetic level induced by ES productinternalization remain relatively poorly understood. Here, we analyze the proteomic changes of two different human cell lines with different responses to *O. viverrini* ES products and reveal novel information about the mechanisms implicated in the internalization of *O. viverrini* ES products and the potential role of this process in the development of CCA. This study is the first step towards a deeper understanding of the mechanisms implicated in the early stages of CCA after *O. viverrini* infection, and could be of importance when designing in vivo experiments aimed at addressing the evolution of CCA by disrupting or blocking the pathways described here.

2. Materials and methods

2.1. Ethics statement

The protocols used for animal experimentation were approved by the Animal Ethics Committee of Khon Kaen University, based on the ethics of animal experimentation of the National Research Council of Thailand (Ethics clearance number AEKKU11/2555). All the hamsters (*Mesocricetus auratus*) used in this study were bred and maintained at the animal facilities at the Faculty of Medicine (Khon Kaen University, Thailand) under the National Experimental Animal Care Guidelines. Fish used in this study were purchased dead at the *O. viverrini* endemic area of Lawa Lake (Khon Kaen, Thailand) and immediately transported on ice to our laboratory at Khon Kaen University to harvest the metacercariae.

2.2. O. viverrini ES product preparation

O. viverrini adults were collected from hamsters 2 months post-infection and ES products harvested as previously described [5,13]. Briefly, 50 *O. viverrini* metacercariae, harvested from naturally infected cyprinoid fish, were used to infect hamsters by stomach intubation. All animals were anesthetized with gaseous carbon dioxide before infection by intragastric intubation and euthanized with gaseous carbon dioxide 2 months after infection. Hamsters were checked daily and no obvious physical signs of morbidity were observed during the experiment. All hamsters remained alive during the experiment (8 weeks). Adult worms were recovered from the bile ducts, washed several times with PBS and cultured in RPMI-1640 (Invitrogen) containing 1% glucose and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin) at 37 °C, 5% CO_2. The ES products were collected twice daily over 3 consecutive days, subsequently pooled, concentrated using a 3 kDa Jumbosep spin concentrator (Pall) and adsorbed with Triton X114 as previously described [16] to remove residual lipopolysaccharide (LPS).

2.3. Cell culture

Human H69 cholangiocytes [17–19] and Caco-2 [20,21] cells were obtained from American Type Culture Collection (Manassas, VA, USA). The H69 cholangiocyte cell line is a SV40-transformed human bile-duct epithelial cell line originally established from a normal liver transplantation [18]. The CaCo-2 cell line (designation HTB-37) is a heterogeneous human epithelial colorectal adenocarcinoma cell line [20]. Caco-2 cells were maintained in T75cm^2 vented monolayer flasks (Corning) with regular splitting using 0.25% trypsin (Life Technologies) every 2–5 days in complete media containing DMEM (Sigma), 10% fetal calf serum (FCS), 1× L-Glutamine, 1× non-essential amino acid (Gibco) and 1× Penicillin–Streptomycin (Gibco) at 37 °C and 5% CO_2. H69 cells (SV40 immortalized cholangiocytes) were grown under similar conditions with growth factor supplemented specialist complete media [18] (DMEM/F12 with high glucose, 10% FBS, 1× antibiotic/antimycotic, 25 μg/ml adenine, 5 μg/ml insulin, 1 μg/ml epinephrine, 8.3 μg/ml holo-transferrin, 0.62 μg/ml, hydrocortisone, 13.6 ng/ml T3 and 10 ng/ml EGF — Life Technologies).

2.4. Sample preparation and protein extraction

H69 and Caco-2 cells were co-cultured with 10 μg/ml of ES products in PBS for 2 h, 6 h, 12 h, 24 h and 48 h, followed by three washing cycles with PBS containing protease inhibitor cocktail. Two biological replicates for each time-point were ground with a TissueLyser II (QIAGEN) in lysis buffer containing 5 M urea, 2 M thiourea, 0.1% SDS, 1% triton X-100 and 40 mM Tris (pH 7.4) using 5 mm stainless beads in 2 ml microcentrifuge tubes at 4 °C for 10 min followed by incubation on ice for 30 min, and centrifugation at 12,000 *g* at 4 °C for 20 min. The pellet was discarded and protein supernatant was subsequently precipitated with 10 volumes of cold methanol at −20 °C overnight, centrifuged at 8000 *g* for 10 min at 4 °C, and allowed to air dry for 5–10 min. A total of two different biological replicates were analyzed for each cell line.

2.5. Protein digestion and iTRAQ labeling

Protein samples were re-suspended in 20 μl of dissolution buffer (0.5 TEAB). Reduction, alkylation, digestion and iTRAQ labeling was performed according to the manufacturer's protocol (AB Sciex). Briefly, each protein sample was denatured with 2% SDS, reduced with 50 mM Tris-(2-carboxyethyl)-phosphine (TCEP) at 60 °C for 1 h, and cysteine residues were alkylated with 10 mM methyl methanethiosulfate (MMTS) solution at RT for 10 min followed by tryptic digestion using 2 μg of trypsin (Sigma-Aldrich) at 37 °C for 16 h. Each sample was labeled with different iTRAQ reagents having distinct isotopic compositions and all samples were subsequently combined into one tube for OFFGEL fractionation and LC–MS/MS analysis.

2.6. Peptide OFFGEL fractionation

Peptide separation based on pI was performed using a 3100 OFFGEL Fractionator (Agilent Technologies) with a 24 well setup. Desalting of samples was performed prior to electrofocusing using a HiTrap SP HP column (GE Healthcare) and a Sep-Pak C18 cartridge (Waters) was used to remove excess of iTRAQ labeling according to the manufacturer's instructions. Briefly, the 24 cm long, 3–10 linear pH range IPG gel strips (GE Healthcare) were rehydrated with IPG Strip Rehydration Solution for 15 min. A total of 3.6 ml of OFFGEL peptide sample solution was used to dissolve the samples and 150 μl was loaded in each well. A maximum current of 50 μA (until 50 kVh was reached) was used for electrofocusing. Every peptide fraction was harvested and each well rinsed with 150 μl of a solution of water/methanol/formic acid (49%/50%/1%) for 15 min. Solutions were pooled with their corresponding peptide fraction and all fractions were evaporated using a vacuum concentrator. Desalting of samples was performed using ZipTip (Millipore) according to manufacturer's protocol followed by centrifugation under vacuum.

2.7. Reverse-Phase (RP) LC–MS/MS analysis

Each dried fraction was reconstituted in 10 μl of 5% formic acid and 3 μl of the resulting suspension was injected into a trap column (LC Packings, PepMap C18 pre-column; 5 mm 300 m i.d.; LC Packings) using an Ultimate 3000 HPLC (Dionex Corporation, Sunnyvale, CA) via an isocratic flow of 0.1% formic acid in water at a rate of 20 μl/min for 3 min. Peptides were then eluted onto a PepMap C18 analytical column (15 cm 75 μm i.d.; LC Packings) at a flow rate of 300 nl/min and separated using a linear gradient of 4–80% solvent B over 120 min. The mobile phase consisted of solvent A (0.1% formic acid (aqueous)) and solvent B (0.1% formic acid (aqueous) in 90% acetonitrile). The column eluates were subsequently ionized using the NanoSpray II of a

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