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

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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* (OvES) 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 OvES 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 OvES 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 OvES. The present study provides the first proteomic analysis to address the molecular mechanisms by which OvES products interact with host cells, and sheds light on the cellular processes involved in *O. viverrini*-induced CCA.

Keywords

Opisthorchis viverrini; Excretory/secretory products; Cholangiocytes; CaCo-2 cells; Proteomics; iTRAQ

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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].

Despite these recent advances, the cellular changes at a proteomic and genetic level induced by ES product internalization 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% CO₂. 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 T75cm² 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% CO₂. 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 QSTAR Elite instrument (Applied Biosystems) operated in information-dependent acquisition mode, in which a 1-s TOF MS scan from 300 to 2000 m/z was performed, followed by 2-s product ion scans from 100 to 2000 m/z on the three most intense doubly or triply charged ions. Analyst 2.0 software was used for data acquisition and analysis.

2.8. Database searching and bioinformatic analysis

Database searches were performed against a subset of the Swissprot database (version 09/2013) containing only proteins from *Homo sapiens* using the MASCOT search engine v4.0 (Matrix-Science). The parameters used were: enzyme; trypsin; precursor ion mass tolerance = ± 0.8 Da; fixed modifications = carbamidomethylation; variable modifications = methionine oxidation and iTRAQ standard modifications; charges states = +2, +3 and allowing for 2 number of missed cleavages. The results from the Mascot searches were validated using the X! Tandem search engine with the program Scaffold Q+ (version Scaffold_4.2.1, Proteome Software Inc., Portland, USA) [22]. Peptide and protein identifications were accepted if they could be established at greater than 95% and 99% probability, respectively, as specified by the Peptide Prophet algorithm [23], and contained at least two identified peptides (proteins) [24]. Proteins containing similar peptides that could not be differentiated based on MS/MS analysis were grouped to satisfy the principles of parsimony. A false discovery rate of <0.1% was calculated using protein identifications validated using the Scaffold Q+ program (v.4.2.1).

Scaffold Q+ (v.4.2.1) was used to quantify the isobaric tag peptide and protein identifications. Channels were corrected in all samples according to the algorithm described in i-Tracker [25] and acquired intensities in the experiment were globally normalized across all acquisition runs. Individual quantitative samples were normalized within each acquisition run, and intensities for each peptide identification were normalized within the assigned protein. The reference channels were normalized to produce a 1:1 fold change. All normalization calculations were performed using medians to multiplicatively normalize data. Differentially expressed proteins were determined using the Kruskal–Wallis test and results expressed as log2 ratios. Only proteins with a *P*-value <0.05 and a significant log2 fold-change >0.6 or <−0.6 (for upregulated and downregulated protein expression respectively) were taken into consideration for further analysis.

Proteins were classified according to gene ontology (GO) categories using the program Blast2Go [26] and Pfam analysis was performed using HMMER (<http://hmmer.org/>). Heatmaps representing the differentially expressed proteins were generated in R using ggplot2 and clustering was performed using Euclidean distances. A cytoscape plugin, the Biological Networks Gene Ontology tool (BiNGO 2.3) was used to identify overrepresented molecular function GO terms [27,28]. Settings for BiNGO included using a hypergeometric test with a significance threshold of 0.05. The *P*-values were corrected for multiple testing by the Benjamini & Hochberg correction. Proteins were compared against the full human

annotation GO database. The Cytoscape plugin ClueGO was used to integrate the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome pathways [28,29]. The enrichment tests for terms and groups were right-sided (Enrichment) tests based on the hyper-geometric distribution with a Kappa Score Threshold of 0.3 and all terms that were significant with $P < 0.05$ (after correcting for multiple testing using the Bonferroni step down false discovery rate correction) were selected for further analysis.

3. Results

A quantitative proteomics analysis was performed to identify the proteins that were differentially expressed on a time-course basis in H69 and Caco-2 cells after incubation with *O. viverrini* ES products. A total of 26,814 and 40,749 MS/MS spectra were acquired in Caco-2 and H69 samples, respectively, from two different biological replicates from each cell line. From these, 4884 and 8754 MS/MS spectra were used to assign unique peptides and unique proteins in Caco-2 and H69 samples, respectively. The average false discovery rate (FDR) for peptide identification was $<0.1\%$ in all samples and was calculated by Scaffold software. To determine which proteins showed altered expression, a Kruskal–Wallis analysis comparing the mean for each protein from the different samples to the control sample was performed. From all the proteins containing a P -value <0.05 only proteins whose expression fold-change was >0.6 or <-0.6 ($\log_2$) were taken into consideration for further analysis. The expression of 142 and 96 proteins was significantly altered in H69 and Caco-2 cells, respectively. A detailed report with all MS/MS identifications and statistical analysis was generated with Scaffold software (S1–S2 Tables).

H69 cell proteins with significantly altered expression levels were grouped into 4 different GO categories (binding, enzymatic activity, structural and other) and a hierarchical clustering was performed to generate a dendrogram and colored heatmap (Fig. 1). Similarly a hierarchical clustering of the 96 proteins with significantly altered expression in Caco-2 cells after incubation with *O. viverrini* ES products was performed to generate a dendrogram and colored heatmap (Fig. 2). A total of 35 proteins from H69 cells (24.6%) presented a significant downregulated expression profile in at least 3 different time points (compared to 15.5% of proteins with an upregulated expression), whereas 37.5% of the proteins from Caco-2 cells presented a significantly upregulated expression profile (compared to 21.8% of proteins with a downregulated expression). The expression of proteins related to “binding” and “structural activity” was up- and down-regulated among all clusters in all samples, while increased numbers of proteins associated with “enzymatic activity” showed upregulated expression levels in Caco-2 cells compared to H69 cells (Figs. 1–2; S3–S4 Tables).

Proteins from H69 and Caco-2 cells with significantly altered expression levels were subjected to a Pfam analysis (Fig. 3). The greatest proportion of the proteins from H69 cells with an altered expression after incubation with *O. viverrini* ES products contained an AAA domain (15 proteins), followed by proteins having a Reovirus sigma C capsid protein motif (7 proteins) and 6 proteins containing an unknown function, a leucine-rich repeat or a RNA recognition motif (Fig. 3a). The Pfam analysis on the proteins from Caco-2 cells incubated with *O. viverrini* ES products returned one major groups containing 10 proteins (RNA

recognition) (Fig. 3b), followed by proteins containing a TCP-1/cnp60 chaperonin family motif or a myosin tail domain among others (Fig. 3b).

Proteins from H69 and Caco-2 cells with a significantly altered expression after incubation with *O. viverrini* ES products were annotated. A significant enrichment of the terms “cellular metabolic process” (7.24% and 8.76%, in H69 and Caco-2 cells respectively), “organic substance metabolic process” (7.18%, 9%) and “primary metabolic process” (7.05% and 9%) was observed within “biological process” (Fig. 4a). Interestingly, the term “response to chemical stimulus” was returned for proteins from H69 cells, whereas this term was not found in proteins from Caco-2 cells. The GO analysis returned 17 and 7 cellular component terms for proteins from H69 and Caco-2 cells, respectively (Fig. 4b). The most prominent terms were “cell part”, “membrane-bounded organelle”, “organelle part”, “non-membranebounded organelle” and “protein complex” in both samples. Interestingly, “membrane part” was a returned term for proteins from H69 cells, while it was not found in proteins from Caco-2 cells (Fig. 4b). The enrichment of GO terms in the molecular function ontology was analyzed and a network was generated (Fig. 5) based on GO hierarchy, with the node size related to the number of proteins associated with a particular GO term and color related to *P*-value for the statistical significance of the enrichment of a GO term. Proteins from H69 cells were grouped into 4 major categories: “catalytic activity”, “structural activity”, “enzyme regulator activity” and “binding activity”, while proteins from Caco-2 cells were grouped in 5 categories (“catalytic activity”, “structural activity”, “enzyme regulator activity” “binding activity” and “transferase activity”). Furthermore, categories returned from Caco-2 cell proteins were more interconnected with nodes belonging to more than one category than those from H69 cholangiocyte proteins (Fig. 5).

The ClueGO plugin in Cytoscape was used to further investigate processes activated in H69 and Caco-2 cells after incubation with *O. viverrini* ES products. Different networks were created based on the overlap between the different KEGG and Reactome categories and the significance. An overview of the KEGG processes induced in H69 and Caco-2 cells can be observed in Fig. 6a–b. Different processes related to metabolism (“glycolysis”, “cysteine and methionine metabolism” and “arginine and proline metabolism”) were common to both cell lines, while the process “protein processing in endoplasmic reticulum” was induced only in H69 cells (Fig. 6a) and “pyruvate metabolism” in Caco-2 cells (Fig. 6b). A summary of the Reactome pathways induced in H69 and Caco-2 cells can be observed in Fig. 6c–d. A substantial number of processes were induced in H69 and Caco-2 cells, with “apoptotic execution phase” and “apoptosis” pathways as the most represented in H69 cells (Fig. 6c) and “regulation of mRNA stability by proteins that bind AU-rich elements” and “activation of chaperone genes by ATF-6 alpha” in Caco-2 cells (Fig. 6d).

4. Discussion

The liver fluke *O. viverrini* secretes a spectrum of diverse proteins from its tegument outer membranous surface and the excretory openings [10], and at least some of these proteins are internalized by host cells lining the biliary epithelium [1,3]. Immunopathology induced by *O. viverrini* ES products is believed to be one of the major mechanisms of pathogenesis induced by the parasite, together with a mechanical irritation and prolonged inflammation

[1,6,8,9]. However, the mechanisms by which ES products are internalized and subsequently induce inflammatory responses and ultimately cancer are not clear. We have performed the first high-throughput quantitative proteomic analysis in two different cell lines that display very different responses and degrees of internalization to *O. viverrini* ES products [14]; moreover, these two human cell types have dissimilar morphological and genetic features such as their tissues of origin, cancerous state and different gene expression profiles including the TLRs [30,31]. While H69 cholangiocytes express all known TLRs, Caco-2 cells fail to express TLR-4, which functionality is impaired in these colorectal cells [30,31]. We hypothesized that if these two cell types displayed very different abilities to internalize *O. viverrini* ES products [14], we could begin to understand the selectivity process of ES internalization by conducting a detailed proteomic investigation of this process over time.

A total of 142 and 96 proteins were identified in H69 and Caco-2 cells respectively with altered expression levels after exposure to ES products. Despite the absence of a clear pattern of up- or down-regulation in any of the studied time-points or any of the groups analyzed, more proteins related to “enzymatic activity” presented an altered expression pattern in Caco-2 cells than in H69 cells. Among all proteins implicated in “enzymatic activity” alpha-enolase was particularly prominent. The expression level of this protein was downregulated in H69 cells at all time-points except at 48 h after incubation with *O. viverrini* ES products, at which point it was significantly upregulated. Interestingly, Yonglithipagon et al. [32] suggested a role for alpha-enolase as a prognostic indicator for CCA patients because the expression level of this protein was upregulated in carcinogenic cells when compared to non-carcinogenic H69 cells. The fact that the expression level of this protein was only upregulated after 48 h could be linked to a change in the cell growth pattern observed for this cell line, but more time-points should be studied to confirm this hypothesis.

Proteins from H69 and Caco-2 cells presenting an altered expression were categorized according to the presence of conserved (Pfam) domains. The most abundantly represented family in H69 proteins were the ATPases associated with diverse cellular activities (AAA domain). One of the main functions of proteins with AAA-domain is related to molecular motion [33], in which dynein plays a key role, however the cytoplasmic dynein 1 heavy chain 1 found in H69 cells was downregulated in all experiments. Furthermore, diverse AAA-ATPases have been found to play roles in fusion and mitochondrial protein translation and degradation, as well as translocation activities [34,35].

A GO analysis of the differentially expressed proteins in H69 and Caco-2 cells displayed some differences in terms belonging to “biological process” and “cellular component” categories. One of the major differences found within “biological process” was the presence of proteins involved in a “response to chemical stimulus” in H69 cells while not in Caco-2 cells. According to the ontology, among this group we found proteins associated with any process that results in a change in state or activity of a cell or an organism in response to chemical stimulus. In this sense, the ES products from the parasite could be stimulating a response in H69 cells as a process of internalization, while not in Caco-2 cells which are less effective at internalizing ES products [14]. Furthermore, an enrichment of proteins belonging to “membrane part” was observed in H69 cells compared to Caco-2 cells. When we analyzed in more detail the terms for “cellular component” we found proteins related to

clathrin, endoplasmic reticulum, Golgi and trans-Golgi and mitochondria terms. These results support previous findings showing that a clathrin-mediated endocytosis pathway might be the main mechanism involved in the internalization of ES products [14]. In addition, a molecular function network analysis for proteins with a significantly altered expression in H69 and Caco-2 cells was performed. Structural activities were more significantly enriched in H69 cells compared to Caco-2 cells, which could be related to morphological changes induced by the ES products from *O. viverrini*. In this regard, the ES products from *O. viverrini* have been shown to modify the morphology of NIH-3 T3 cells to a refractive and narrow shape allowing the cells to proliferate in a limited culture area [36].

A KEGG and Reactome pathway analysis was performed on the proteins from H69 and Caco-2 cells with a significant altered expression using the ClueGO plugin in Cytoscape. Two major processes were altered in H69 cells compared to Caco-2 cells: glycolysis/gluconeogenesis and protein processing in the endoplasmic reticulum. It is known that the parasite contains genes associated with anaerobic and aerobic glycolysis, and that it uses glucose for energy production [12]. In addition, elevated proliferation of the smooth endoplasmic reticulum has been observed in the hepatocytes of animals infected with *O. viverrini* [37]. This organelle is involved in the production of lipids and steroid hormones by cells, and enables glycogen that is stored as granules on the external surface of smooth ER to be broken down to glucose. The ES products might be activating the breakdown of glycogen in H69 cells into glucose monomers via glycolytic enzymes so parasites can find more glucose to consume. The internalization of ES products is likely reduced in Caco-2 cells because of their impaired TLR-4 functionality [14,15], and these cells would not be experiencing alterations in the glycolytic or ER pathways due to the reduced internalization of *O. viverrini* ES products. However, it has been shown that TLR4 plays a key role in glucose metabolism [38,39]. Despite the upregulation of glycolytic pathways observed in H69 cells after internalization of *O. viverrini* ES products, the absence of glycolysis upregulation in Caco-2 cells could be related to the impaired functionality and reduced presence of TLR-4 receptors in this cell type, although more work should be done to further address this point. It is tempting to speculate that the parasite has adapted to migrate from the intestine to the bile ducts to find a more suitable niche where it can more readily obtain glucose. The Reactome pathway analysis showed a domination of the pathways related to apoptosis and the apoptotic execution phase in the proteins from H69 cells that presented an altered expression after incubation of cells with *O. viverrini* ES products. This finding agrees with previous studies that identified ES proteins with growth factors and anti-apoptotic properties [10,40,41]. Recently, Matchimakul et al. showed that thioredoxin, a component of the ES products, downregulates expression of apoptotic genes and upregulates expression of anti-apoptosis-related genes including different caspases and signaling kinase 1 [40]. In addition, granulin, a growth factor present in the ES products of *O. viverrini* has been demonstrated to induce angiogenesis and wound repair in mice by altering the expression of proteins associated with wound healing and cancer pathways [41].

We have characterized the immediate proteomic changes occurring in cholangiocytes after *O. viverrini* ES internalization. The understanding of these processes can shed light on the immunopathogenesis of this carcinogenic infection and provide new insights to facilitate the development of new strategies to combat this carcinogenic liver fluke.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Clustered heatmap of the H69 cell proteins presenting a significantly altered expression after *Opisthorchis viverrini* excretory/secretory product incubation. Proteins were grouped into 4 different GO categories based on GO annotation and clustering was performed using Euclidean distances.

Fig. 2.

Clustered heatmap of the Caco-2 cell proteins presenting a significantly altered expression after *Opisthorchis viverrini* excretory/secretory product incubation. Proteins were grouped into 4 different GO categories based on GO annotation and clustering was performed using Euclidean distances.

Fig. 3. Pfam analysis comparison. Donut chart summarizing protein family (Pfam) abundance in the significantly regulated proteins from H69 cells (A) and Caco-2 cells (B) after incubation with *Opisthorchis viverrini* excretory/secretory products. Only ten most abundant families were represented in the chart.

Fig. 4. Gene ontology analysis. Bar graph showing the most abundantly represented gene ontology (GO) biological function (A) and cellular component (B) terms in H69 cells and CaCo-2 cells after incubation with *Opisthorchis viverrini* excretory/secretory products.

Fig. 5. Molecular function networks. Molecular function networks for proteins with significantly altered expression levels in H69 (A) and Caco-2 cells (B) after incubation with *Opisthorchis viverrini* excretory/secretory products. Color relates to the *P*-value for the statistical significance of the enrichment of a GO term, while node size is related to the number of proteins associated with a GO term. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. KEGG and Reactome pathway analysis. The Cytoscape plugin ClueGO was used to analyze the KEGG pathways induced in H69 (A) and Caco-2 (B) cells after incubation with *Opisthorchis viverrini* excretory/secretory (ES) products and Reactome pathways enriched in H69 (C) and Caco-2 (D) cells after incubation with *O. viverrini* ES products.