Genomics of worms, with an emphasis on *Opisthorchis viverrini* - opportunities for fundamental discovery and biomedical outcomes

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ABSTRACT

Neglected tropical diseases cause substantial morbidity and mortality in animals and people globally. Opisthorchiasis is one such disease, caused by the carcinogenic, Asian liver fluke, *Opisthorchis viverrini*. This hepatobiliary disease is known to be associated with malignant cancer (cholangiocarcinoma, CCA) and affects millions of people in Asia, including Thailand, Lao People's Democractic Republic (PDR) and Cambodia. No vaccine is available, and only one drug (praziquantel) is routinely employed against the parasite. Relatively little is known about the molecular biology of the fluke itself and the disease complex that it causes in humans. With the advent of high-throughput nucleic acid sequencing and bioinformatic technologies, it has now become possible to gain global insights into the molecular biology of parasites. The purpose of this plenary talk was (i) to discuss recent progress on the genomics of parasitic worms, with an emphasis on the draft genome and transcriptome of *O. viverrini*; (ii) using results from an integrated, global analysis of the 'omic data, to explain how we believe that this carcinogenic fluke establishes in the biliary system, how it feeds, survives and protects itself in such a hostile, microaerobic environment within the liver, and to propose how this parasite evades or modulates host attack; and (iv) to indicate some of the challenges, and, more importantly, the exciting opportunities that the 'omic resources for *O. viverrini* now provide for a plethora of fundamental and applied research areas. Looking ahead, we hope that this genomic resource stimulates vibrant and productive collaborations within a consortium context, focused on the effective control of opisthorchiasis.

Keywords:
Neglected tropical disease (NTD)
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1. Introduction

Compounded by a massive global food shortage, neglected tropical diseases (NTDs) caused by parasitic worms are amongst the world's big challenges (London Declaration, 2012). Billions of people are infected with worms (= helminths), which have a comparable socio-economic burden to that of diabetes or lung cancer in disability adjusted life years (DALYs) (WHO, 2004). These worms include roundworms (= nematodes) and flatworms (= flukes and tapeworms).

Liver flukes, such as *Clonorchis* and *Opisthorchis* spp. (family Opisthorchiidae), are particularly important food-borne pathogens of humans and other fish-eating mammals including canids and felids (Lun et al., 2005; Keiser and Utzinger, 2009). These parasites are particularly notable because they are classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC; Bourvard et al., 2009). *Opisthorchis viverrini* causes opisthorchiasis, which has a major public health impact mainly in countries of the Asia Pacific (Keiser and Utzinger, 2009; Sripa et al., 2007). This pathogen affects tens of millions of people, and millions more are at risk of reccurrent infection (Sripa et al., 2007; Keiser and Utzinger, 2009). Despite control efforts, disease prevalence can be as high as 70% in some Asian countries including Thailand (e.g., Sripa et al., 2007; Sithithaworn et al., 2012). Chronic infection is linked to cholangitis, bile duct cancer (= cholangiocarcinoma, CCA) and associated complications (Sripa et al., 2007; Shin et al., 2010). Although CCA incidence is low in Western countries, this cancer is prevalent in many parts of South East Asia where *O. viverrini* is endemic, including Cambodia, Lao PDR and Thailand, where an age-standardised incidence rate (ASIR) of up to 96 per 100,000 has been reported (Khan et al., 2008). Current estimates indicate that chronic opisthorchiasis affects ~10 million people worldwide, and, in Asia, fluke-associated CCA is detected in > 2,500 people annually (Parkin, 2006). The control of opisthorchiasis relies principally on treating infected people with praziquantel (an anthelmintic compound), as the cultural tradition of eating raw cyprinoid fish (second intermediate host infected with metacercariae) is entrenched. Humans develop only a limited degree of immunity against opisthorchiid liver flukes (Wongratanacheewin et al., 2003), such that they frequently become re-infected. Although there has also been a focus on developing alternative control methods (Keiser et al., 2006, 2009), no vaccines are yet available. In our opinion, detailed fundamental molecular biological investigations of *O. viverrini*/opisthorchiasis could be useful to underpin the development of novel intervention methods. However, until recently, most molecular studies of flatworms had focused predominantly on human blood flukes (schistosomes) (Berriman et al., 2009; *Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium, 2009; Young et al., 2012), complemented by sustained efforts to establish *in vitro* systems for functional genomic analyses (e.g., Brindley and Pearce, 2007; Hagen et al., 2011; Sripa et al., 2011).

In 2009, when the genomes of two blood flukes (*Schistosoma mansoni* and *S. japonicum*) were published (Berriman et al., 2009; *Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium, 2009), there was very little molecular information available for opisthorchid flukes. In 2010, we undertook the first transcriptomic surveys of *C. sinensis* and *O. viverrini* using 454 sequencing technology (Young et al., 2010a,b), followed by a study of differential transcription between immature and adult *O. viverrini* (see Jex et al., 2012). To do this, we combined previous 454 sequence data with RNA-sequence (RNA-seq) data to achieve an enhanced assembly and annotation of the transcriptome, in order to underpin the differential transcription analysis. In spite of this enhancement, there were some issues relating to transcript redundancy, incomplete annotations and suboptimal mapping of transcripts. In order to overcome these limitations, in 2012, we decided to sequence the nuclear genome of *O. viverrini* using Illumina technology, at a time when this technology was revolutionizing the sequencing of many other animal genomes (cf. Mardis et al., 2008; Koboldt et al., 2013). With the completion of this draft genome (Young et al., 2015), our intent was to provide the scientific community with a molecular resource for future transcriptomic, proteomic and functional genomic and a plethora of other studies of a carcinogenic liver fluke. In 2011, a draft genome of *C. sinensis* was reported

from China (Wang et al., 2011), and an enhanced version presented by the same research team two years later (Huang et al., 2013).

2. The draft genome of *O. viverrini*, its features and functional annotation

In 2014, our international team published this draft genome (Young et al., 2014); here, we summarise some salient features. We generated 79.9 Gb of short-read sequence data (>130-fold genome coverage) from seven libraries (170 bp to 20 kb) constructed from genomic DNA from multiple adult specimens of *O. viverrini*. Following the verification of low sequence heterozygosity within and among some libraries, we assembled the genome into scaffolds to produce a draft genome of 634.5 Mb (N50 = 1,323,951 bp; repeat content: 31%; GC-content = 44%), in which we found ~86% of 248 core essential genes. We predicted 16,379 protein-encoding genes using transcriptomic evidence and sequence data for *C. sinensis* (see Huang et al., 2013) and blood flukes (Berriman et al., 2009; *Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium, 2009; Young et al., 2012). Most (87%) genes were supported by published RNA-seq data (Young et al., 2010a; Jex et al., 2012), and > 99% of assembled transcripts mapped to the genome. The estimated number of genes, proportion of coding regions (3.4%), and mean total gene length (18,231 bp), exon length (254 bp), intron length (3,531 bp), and mean number of exons per gene (6) were similar to *C. sinensis* (Huang et al., 2013), but distinct from other flukes.

Structurally, the *O. viverrini* draft genome is very divergent from all other published genomes of flukes, including *C. sinensis*, *S. haematobium*, *S. japonicum* and S. *mansoni* (see Berriman et al., 2009; *Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium, 2009; Young et al., 2012; Huang et al., 2013). In particular, only 22% of *O. viverrini* scaffolds could be aligned to 26% of *C. sinensis* scaffolds (at the nucleotide level). We interpreted this lack of synteny to relate to karyotypic differences, with *O. viverrini* having 12 chromosomes (2n) Kaewkong et al., 2012), and *C. sinensis* having 14 (Russian isolate) (Zadesenets et al., 2012) or 58 (Korean isolate; Park et al., 2000), and all human schistosomes having eight chromosomes (Short and Menzel, 1960).

3. What can we learn about this carcinogenic fluke by inference from 'omic and published information?

Although the main morphological changes that take place during the life cycle of *O. viverrini* are well known, little is understood about the molecular and biochemical processes underlying developmental changes and survival as well as parasite-host interactions and disease. Insights into these fundamental processes are critically important, and could provide a basis for the identification of targets for the design of new interventions. Using advanced bioinformatic pipelines for parasites, we integrated all of the genomic, transcriptomic, inferred proteomic data as well as published information to characterize some of the molecular landscape of *O. viverrini* (see Young et al., 2014). This effort allowed us to address some key biological questions regarding fundamental molecular biology of this pathogen, infer essential pathways associated with the fluke-host interplay and to suggest some genes/gene products that might contribute to CCA development:

3.1. How does the fluke migrate to and establishment in the biliary duct?

When ingested, metacercariae of *O. viverrini* pass through the digestive tract, where they excyst, migrate to and then establish in the biliary tract (Nithikathkul et al., 2007). Proteases, including aspartic and cysteine peptidases, appear to play a key role in excystment (Young et al., 2014), being reflected in high transcription in metacercariae (Yoo et al., 2011). The large number of GPCRs and ion channels encoded in the genome might enable chemotaxis-mediated

migration to the biliary duct; molecules such as the rhodopsin biogenic amine receptors and ion channels are conserved for opisthorchiids and divergent from flukes that live external to the biliary system (cf. Young et al., 2014).

3.2. How does the worm feed and nourish itself?

The newly excysted juvenile (NEJ) stage of *O. viverrini* relies initially on energy stored within glycogen granules and lipid droplets in the excretory bladder of the worm (Orido et al., 1990). However, these energy reserves are rapidly depleted once the fluke reaches the bile duct. Thus, the developing fluke must rapidly acquire nutrients and energy from its surrounding environment for survival, development and reproduction. Because bile contains extremely low levels of glucose (Maysuk et al., 2002), the fluke cannot rely on this sugar for energy. However, bile is rich in high (HDLs), intermediate (IDLs), low (LDLs) and very-low density lipoproteins (VLDLs), which all contain differing proportions of triglycerides, phospholipids, cholesterol and amphipathic proteins (Ginsberg, 1998); it is also rich in branched-chain amino acids (Fölsch and Wormsely, 1977) and long-chain saturated palmitic acid (C16:0) as well as unsaturated linoelaidic (C18:2) and arachidonic (C20:4) fatty acids (Coleman, 1987; Halpern et al., 1993).

Therefore, the fluke produces enzymes and accessory proteins to process these bile constituents (cf. Young et al., 2014). Using its vast array of peptidases with broad substrate specificity, the fluke likely degrades lipoprotein complexes and proteins. Free, bile-derived amino acids are then taken up via amino acid transporters, after which they are processed for energy via acetyl CoA (Young et al., 2014). As the fluke is unable to synthesise cholesterol, the fluke likely uses a scavenger-like receptor (SR-B1) to transport cholesterol from HDLs into cells (Acton et al., 1996), and LDL receptor (LDLR), LDLR-related protein 1 receptor (LRP1) and CD36 -like receptors for LDL, IDL and fatty acid uptake, respectively. In addition, the fluke also has an expanded group of lipid-binding proteins (n =25) with a MD-2-related lipid-binding domain, which are homologous to the human Niemann-Pick C2 protein (NPC2; Inohara and Nunez, 2002) and enable intracellular and extracellular sterol transport (Inohara and Nunez, 2002; Berger et al., 2005). Fifteen of these 25 NPC2-like proteins appear to be expressed in fluke stages within the bile duct (Young et al., 2015). Remarkably, other eukaryotes appear to express only one such protein (Inohara and Nunez, 2002). Clearly, this expansion in *O. viverrini* appears to reflect the significance of the binding and/or transportation of sterols and/or lipids and intracellular cholesterol. In addition to an adaptation to a lipid-rich diet, *O. viverrini* also likely degrades cholangiocyte components using galactosylceramidase/galactocerebrosidase (GALC) and sphingomyelin phosphodiesterases (SMPDs), which (within lysosomes) catabolize sphingomyelin, a highly enriched constituent of cholangiocytes (Amigo et al., 1999). In summary, *O. viverrini* has an extensive repertoire of enzymes and receptors required for the absorption, binding, transport and/or conversion of nutrients originating almost exclusively from bile and the biliary epithelium.

3.3. How does the worm survive in the duct and protect itself?

Like many other flukes (Takamiya et al., 2010), *O. viverrini* is a facultative anaerobe within the host, transcribing genes linked to anaerobic (including phosphoenolpyruvate carboxykinase) and aerobic (such as pyruvate kinase) glycolysis (van Hellemond et al., 2003). As oxygen levels are often low in the bile duct (Brook, 1989), haemoglobin of this fluke has a very high oxygen affinity (Kiger et al., 1998). Bile induces cellular stress by generating free oxygen radicals through lipid peroxidation (Lechner et al., 2002), and cholangiocytes and the fluke can be exposed to toxins, drugs and their metabolites (xenobiotics) and carcinogens excreted in bile (Trauner and Boyer, 2003). Inside the bile duct, the fluke likely protects itself using a repertoire of antioxidants, such as intra- and extra-cellular superoxide dismutases (SODs), which convert free radicals to hydrogen peroxide, and *via* glutathione-S-tranferases (GSTs) to reduce lipid

hydroperoxides and detoxify xenobiotic substrates (Trauner and Boyer, 2003). Indeed, secretory GSTs are expressed in the fluke in response to human bile (Bae et al., 2013). Like other flukes (Sanchez-Moreno et al., 1987), *O. viverrini* lacks catalases, and employs glutathione-like and peroxiredoxin peroxidases to convert hydrogen peroxide to water.

3.4. How does the worm evade host attack?

Excretory/secretory (ES) proteins encoded in *O. viverrini* likely play a major role in the evasion or modulation of the host immune responses against the parasite in the biliary system. For example, genes encoding secretory helminth defence proteins (e.g., T265_13308) are highly transcribed in both juvenile and adult stages. In flukes, these proteins are proposed to mimic cathilicidin (= mammalian host defense peptide) and subvert a Th1 response by blocking LPS interaction with the Toll-like receptor 4 complex on macrophages (Robinson et al., 2011). Moreover, cathepsins F likely degrade some immunoglobulins, such as IgA (Kang et al., 2010; Coleman et al., 1987). These proposals warrant detailed study for *O. viverrini*.

ES proteins from *O. viverrini* also likely contribute to fluke-host interactions more generally and CCA development (e.g., Mulvenna et al., 2010). From the genomic and transcriptomic data, we inferred a secretome of 437 proteins (Young et al., 2014); 184 of these molecules (42.1%) were similar to those reported previously for *O. viverrini* and other flukes (cf. Mulvenna et al., 2010). Key examples include heat shock proteins (HSPs), peptidases, superoxide dismutase (SOD) and venom allergen-like (VAL) proteins as well as cysteine peptidases (CatB, AEP), repetin, TP, Niemann-Pick C2 (NPC2) and vitelline B and glutathione transferase (GST) and progranulin (*Ov*-PGRN) and granulins (Young et al., 2014).

Interestingly, *Ov*-PGRN is likely required for endogenous regulation of cell growth, development and maintenance based on evidence for its human homolog (Bateman et al., 1998), and *Ov*-GRN1 is a growth factor, whose gene is highly transcribed in juvenile and adult stages, that stimulates cell proliferation and likely contributes to tumour growth (Smout et al., 2009, 2011; Papatpremsiri et al., 2015). Interestingly, we also discovered *Ov*-GRN2, a novel single-domain granulin which shares high sequence homology to *Ov*-GRN-1 and to *Ov*-PRGN in the granulin domains (Young et al., 2014). Although the function of *Ov*-GRN-2 is presently unknown, differences in the amino acid sequence in the N- and C-termini indicate variation in receptor-binding specificity (Bateman et al., 1998). Given the known agonistic or antagonistic regulation of cell growth by human granulins (Bateman et al., 1998), future studies should compare the mitogenic potential of *Ov*-GRN-2 and *Ov*-GRN-1. In addition, the oncogenic potential of other parasite molecules, such as cathepsins D (cf. Benes et al., 2008), might also be explored.

4. Conclusions

We believe that the major progress in genomics, transcriptomic and bioinformatics, together with the completion of the draft genome and transcriptome of *O. viverrini*, provides a sound foundation to facilitate a plethora of fundamental and applied research areas.

From a fundamental perspective, current resources could now guide or support investigations into the function of genes and gene products. This is particularly pertinent, given that published evidence shows that RNAi works well in *O. viverrini* (see Sripa et al., 2011; Thanasuwan et al., 2014; Papatpremsiri et al., 2015), although it will be advantageous to define the RNAi machinery of *O. viverrini* in the near future. For instance, it will be particularly interesting to learn more about some of the molecules (GRN2) and orphan ('hypothetical') proteins and their possible role(s) in CCA, but also molecules involved in growth, development, reproduction, host-parasite interactions and parasitism. In addition, the genomic resource should provide a solid context for population genetic studies of *O. viverrini*, as it is now possible to deeply sequence genomic DNAs from large numbers individual flukes (at relatively low cost) and undertake bioinformatics

sequence comparisons of thousands of single-copy orthologous genes across the genome. In this way, it would be possible to conduct phylogenetic analyses of thousands of concatenated sequences from hundreds of individuals, to comprehensively explore genetic relationships both within and among distinct populations of *O. viverrini* (e.g., in Thailand). Such an investigation might evaluate further whether distinct subpopulations of *O. viverini* exist that are more likely to cause cancer than others, as there is already evidence that cryptic species exist within *O. viverrini* in Thailand and Lao PDR (Sithithaworn et al., 2012). Although numerous studies have already been conducted in this direction using multilocus enzyme electrophoresis and DNA-based methods (Sithithaworn et al., 2012), they have used relatively small numbers of genetic loci. In addition, genome-wide comparisons using a 'sliding window' analytical approach would allow the identification of genes or gene regions that are variable and conserved in sequence, enabling the definition of a wide array of genetic markers for by PCR-coupled population genetic and/or diagnostic applications.

From an applied perspective, integrated systems biology explorations of the fluke might also guide the design of novel strategies for parasite intervention (e.g., drugs and/or vaccines). This focus is important, given the current reliance on praziquantel for community-based treatment and a possibility that resistance emerges against this compound. For instance, future work might focus on defining a spectrum of key molecules involved in key pathways linked to the development of the nervous system in different stages, and assessing their potential as drug targets. Moreover, exploring select groups of molecules, such as kinases, phosphatases, GPCRs, and the complex array of peptidases, and understanding their roles in host-parasite interactions might also support applied research focused on combatting this fluke and the insidious disease complex that it causes.

In conclusion, we hope that the genomic/transcriptomic resources established for *O. viverrini* (see Young et al., 2010a,b, 2014; Jex et al., 2012) will be useful to scientists around the world working on the fluke, opisthorchiasis and/or CCA and also to those working on translational research focused on biomedical and biotechnological outcomes to prevent and control this insidious disease problem. We concede that there are some challenges in relation to skills required for the handling and bioinformatic analyses of digital data sets, and also regarding research funding for this area. However, we strongly believe that there is major scope for solid and productive collaborations in this area, within the intended Consortium context (Ogorodova et al., 2015), focused also on achieving at least some of the goals set in the London Declaration (2012).

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