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Banking on the future: biobanking for “omics” approaches to biomarker discovery for *Opisthorchis*-induced cholangiocarcinoma in Thailand

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Abstract

Cholangiocarcinoma (CCA) – bile duct cancer - is associated with late presentation, poses challenges for diagnosis, and has high mortality. These features highlight the desperate need for biomarkers that can be measured early and in accessible body fluids such as plasma of people at risk for developing this lethal cancer. In this manuscript, we address previous limitations in the discovery stage of biomarker(s) for CCA and indicate how new generation of “omics” technologies could be used for biomarker discovery in Thailand. A key factor in the success of this biomarker program for CCA is the combination of cutting edge technology with strategic sample acquisition by a biorepositories.

Keywords

Biomarkers; cholangiocarcinoma; liver fibrosis; *Opisthorchiasis*

Cholangiocarcinoma from *Opisthorchis viverrini*

At least 750 million people, i.e. 10% of the human population, are at risk of food-borne trematodiasis, with more than 40 million people currently infected [1]. *O. viverrini* is considered among the most important of the food-borne trematodes due to its strong association with bile duct fibrosis and cholangiocarcinoma (CCA). As determined by the WHO's International Agency for Research on Cancer, no stronger link between a human malignancy and a parasitic infection exists than between CCA and infection with *O. viverrini* [2]. An estimated 10 million people are infected with *O. viverrini* in Thailand and Lao PDR, where uncooked cyprinoid fish, intermediate hosts for *O. viverrini*, are a staple of the diet³. While the infection can be eliminated by the use of the anthelmintic praziquantel (PZQ), environmental and cultural factors of this region strongly favor routine re-infection

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[3]. Accordingly, residents of *O. viverrini* endemic areas in Thailand can remain infected for a lifetime, with 25% of *O. viverrini*-infected individuals developing advanced periductal fibrosis (a precursor of cancer) and less than 1% progressing to CCA [4].

CCA has a worldwide distribution that accounts for about 10–15% of all cases of primary hepatobiliary malignancy [5, 6]. Cholangiocarcinomas are slow-growing tumors that spread longitudinally along the bile ducts with neural, perineural and subepithelial extensions and may metastasize to distant organs via the lymphatic and/or vascular systems [7–9]. While in Western countries, 90–95% of cholangiocarcinomas arise in the extrahepatic ducts, in countries where *O. viverrini* is endemic (Thailand, Lao PDR, etc), intrahepatic and extrahepatic CCA account for 40% and 60% of all cases respectively, with the majority of cases identified at the proposed study site (Khon Kaen, Thailand) being intrahepatic [4, 10–12]. The location of these neoplasms in the upper hepatoduodenal ligament and their extension into the liver and their proximity to major vascular structures makes early evaluation of CCA challenging, hence, the need for the development of a non-invasive biomarker assay for risk assessment and early detection [8]. The prognosis of patients with unresectable CCA tumors is poor, with mortality within a year of diagnosis a common outcome [13]. Beyond palliative therapy, medical treatment for CCA is unavailable, and surgery and supportive treatment are complicated and often not accessible to individuals with CCA in resource poor-settings such as rural Thailand. Other risk factors for CCA include primary sclerosing cholangitis, hepatolithiasis, and choledochal cysts [13]. Most of these factors share long-standing inflammation and chronic injury of the biliary epithelium as common determinants associated with *O. viverrini*-linked CCA [4, 14].

BOX 1

DEVELOPING BIOMARKERS FOR CCA

1. **The strong association between *O. viverrini* infection and CCA** enables the assessment of biomarkers in individuals at risk of CCA [2, 4, 6].
2. **The highest incidence of intrahepatic CCA in the world** enables the study of large collections of banked tissue and plasma from confirmed CCA individuals and individuals at risk of *O. viverrini* related CCA [2, 4, 6].
3. **Biomarker discovery proximal to the tumor site** enables a direct measurement of protein expression in the source tissue before verification in plasma [15].

IMPACT OF DEVELOPING BIOMARKERS

CCA is associated with a late presentation, poses challenges for diagnosis and has high mortality rate - features that highlight the need for biomarkers than can be measured early and in accessible samples such as plasma [16]. However, despite extensive investigations to date, efforts have failed to yield biomarkers with adequate diagnostic accuracy and utility for CCA in plasma.[16]

The task of CCA biomarker discovery outside of Thailand is complicated and challenging because:

1. The cause of CCA (outside of East Asia) remains obscure [4, 8, 17].
2. Investigators lack well-defined cohorts of individuals at risk of CCA in whom biomarkers could be ascertained and verified for their diagnostic or prognostic value.

3. The relative rarity of this form of liver cancer outside of East Asia and hence the small sample sizes for biomarker discovery [18].
4. Biomarker discovery programs have focused on mining complex matrices such as serum and plasma for biomarkers [16].

For “omics” approaches strategic biobanking is the key

In recent years, biobanks of human tissues have evolved from small-scale collections of pathological materials into structured resource centers for the acquisition, storage, processing, and usage of high-quality biospecimens for research[19]. This evolution goes hand in hand with the development of highly sensitive, high-throughput “omics” methods for biomarker discovery[19]. Recent advances in high-throughput assays for gene expression (genomics), proteins (proteomics), and metabolites (metabolomics) have engendered a parallel need for well-annotated human biological samples--indeed, human tissue biobanking is a critical component to the success of any “omics” based biomarker research program[19, 20]. Importantly, biospecimen collection for omics such as proteomics or microRNA requires unique sample collection strategies [19, 20]. Listed below are the two ideal sample collection strategies necessary for an omic program on *O. viverrini*-induced CCA in Thailand.

1. Frozen primary tumor and unaffected tissue from resected liver specimens from confirmed CCA cases [19, 20].
2. Collection of plasma or serum samples drawn simultaneously as the frozen tissue samples above.

Note that Formalin-Fixed Paraffin-Embedded (FFPE) tissues should also be sorted but more for immunohistochemistry as many omics’ based assays, especially proteomics methods [19, 20], FFPE samples presents problems. However, recent advances in microRNA have shown FFPE samples to be of value. To achieve even these simple collection strategies, it is essential to perform innovative research on improving all aspects of specimen processing, including the development of quality controls applicable to retrospective collections. This requires a dedicated effort from funding agencies and from the scientific and medical publication community. Training of highly qualified tissue-banking professionals would increase the standards of biobanking as well as the recognition of biobanking as an integral part of research--that is, the first step in a good omics research program [19, 20]. It would also facilitate the development and dissemination of a corpus of harmonized, evidence-based tissue-banking procedures [19, 20]. In addition to this research role, the use of cellular and molecular biomarkers is rapidly becoming a standard part of hospital pathology practice and of therapeutic decision schemes [19, 20]. Hence, biobanking for omics may be a key mechanism for translating newly discovered biomarkers into clinical practice [19, 20]. Furthermore, biobanking is speculated to become an intrinsic part of pathology requirements in the context of standard clinical care.

An “omics” approach starts with cell line work

The objective of a biomarker program should be on frozen, resected liver tumor tissues and matched plasma from confirmed liver fluke-induced CCA patients to determine a suite of proteins (candidate biomarkers) proximal to the disease site. However, a novel “training set” method is to complement research on banked tissue and plasma with a similar analysis of CCA cell lines. Because of the relative abundance of protein material from cell lines, they provide a reliable assay for measuring protein expression variation. Moreover, unlike frozen tissue sections, it is possible to measure the relative expression of secreted/membrane proteins (secretome) in culture media. Potential biomarkers identified during the analysis of cell lines could also be verified in the plasma samples from confirmed *O. viverrini* CCA

patients. The candidate biomarkers could also be verified in the plasma of healthy and at-risk individuals, resident in areas of high transmission of *O. viverrini* along the Che River Basin.

O. viverrini CCA is an excellent model for omics approaches; The use of cutting-edge proteomic methods on the unique set of biobanked samples that could be stored in Khon Kaen would overcome the previous limitations in CCA biomarker discovery in the following manner:

First: The OV-induced CCA model—Whereas the causative agent for most cancers, including CCA in the West, remains obscure, the single most important risk factor for intrahepatic CCA in Thailand has long been established – infection with the liver fluke *O. viverrini* [11]. This well-established link between infection and cancer (CCA) should be utilized for the discovery and verification of biomarkers for cholangiocarcinoma. In this regard, the **OV-induced CCA model** offers an exceptional opportunity to study biomarkers for cholangiocarcinogenesis.

Second: Ample clinical samples for biomarker discovery and verification—A persistent limitation in the search for CCA biomarkers is the limited incidence of CCA [13]. In the West, CCA is an uncommon neoplasm, accounting for approximately 3% of all gastrointestinal malignant disease [13]. This results in limited clinical samples for biomarker discovery and almost no samples for verification and validation of biomarkers in healthy individuals at risk for CCA. However, Khon Kaen Province Thailand has the highest incidence of CCA in the world: 98 per 100,000 which can be compared to 0.95 per 100,000 for intrahepatic forms and 0.82 for per 100,000 for extrahepatic forms of CCA in the Ultrasound [4]. By way of further comparison, CCA is responsible for about 19% of liver cancers in the United States, compared with 71% in Khon Kaen, Thailand [4]. The incidence of CCA in the four major regions of Thailand varies by at least 12-fold and has a strong positive correlation with the prevalence of *O. viverrini* infection. These represent an unparalleled resource of clinical samples for the discovery phase of this biomarker program[4].

Third: Disease progression from chronic *O. viverrini* infection to CCA—During the decades of *O. viverrini* infection, a continuum of clearly-defined clinical events--starting with bile duct inflammation, proceeding through advanced periductal fibrosis, and, in some cases, concluding with a diagnosis of CCA--has been mapped out in our longitudinal study of OV pathogenesis (Figure 1) [4, 17]. As with other chronic inflammatory disorders, persistent irritants sustain the simultaneous production of growth factors and fibrogenic cytokines, which in turn stimulate the deposition of connective tissue elements that progressively remodel and destroy the normal tissue architecture of the biliary epithelium, resulting in the accumulation of fibrotic elements. Advanced periductal fibrosis (APF) provides the basis for malignant transformation to CCA and is readily diagnosed by ultrasonography [11, 14, 21, 22]. This process has been confirmed in an animal model of CCA, which closely parallels the proposed carcinogenic processes in humans; after only 12 weeks of infection, the chronically inflamed biliary epithelium of the *O. viverrini*-infected hamster shows advancing fibrosis throughout its length. More importantly, as with humans, fibrotic deposition in the biliary epithelium is considered the most important precursor event to CCA in the hamster model [23].

Figure 1 shows the diseases stages for proteomic discovery and verification for a biomarker for *O. viverrini* related CCA, which specifically includes paired liver tumor tissue (resected) and plasma taken from confirmed CCA patients, as well as plasma from individuals at every stage along the disease continuum shown here. Together, this biobanked material provides a

powerful resource for discovery and verification of the presence of proteins directly related to CCA.

Box 2

Opisthorchiasis results in well-defined, progressive clinical stages

During chronic opisthorchiasis, pathogenesis is manifested in a series of well-defined stages detected by common clinical methods:

- *O. viverrini* infection detected through fecal exam
- Bile duct inflammation detected by serum levels of IL-6 [24].
- Advanced periductal fibrosis detected by ultrasound (US) [25, 26].
- Hepatobiliary abnormalities such as loss of gall bladder contractility, gallbladder sludge, etc as detected by US [25, 26]
- Early CCA detected by US [9, 24, 26]

Fourth: Targeted biomarker discovery proximal to the tumor site—The selection of candidate biomarkers is often complicated due to the analyzed sample type. Plasma is the easiest available source for diagnostics – hence, most proteomic programs for biomarker discovery start by mining the plasma proteome [16]. However, plasma and serum exhibit an enormous dynamic range in protein abundance and are extremely complex matrices for identifying new biomarkers with non-targeted proteomic analytical strategies. Hence, the selection and analysis of the affected cell type (i.e. CCA tumor tissue) is fundamental for a focused identification of new candidate biomarker with high specificity for a given disease. Therefore, the initial stage of biomarker discovery program should be to reduce the number of candidate biomarkers. By applying quantitative proteomic techniques, candidate biomarkers can be identified at their place of activity (primary biliary tumors) and subsequently their presence can be verified in paired plasma samples. Moreover, candidate biomarkers are more abundant at their place of origin and once known can be detected and quantified by targeted proteomic analyses in complex matrices such as plasma.

INNOVATION

While the causative agent for most cancers, including CCA in the West, remain obscure, the single most important risk factor for intrahepatic CCA in Thailand has long been established – infection with the liver fluke *O. viverrini*. In the proposed studies, we plan to utilize this well-established (yet little studied in the West) link between infection and cancer for the discovery and verification of biomarkers for CCA. Thus, **the overarching innovation** of such studies is that they can utilize a model of human carcinogenesis, in which the major risk factor as well as many of the intermediate stages on the pathway to cancer have been well-defined to track the presence of biomarkers from the initiation of risk to its culmination in CCA [4, 12, 14, 17]. Specifically, the study of frozen liver sections from primary biliary tumors, from *O. viverrini* induced CCA, which could be utilized for biomarker discovery. In parallel, the ideal omics approach would utilize established CCA cell lines to examine the both the secretome and the membrane-associated (peripheral and integral) proteins for potential biomarkers. When combined with matched plasma samples, these clinical samples represent an innovative, compelling, and indeed invaluable resource for CCA biomarker discovery, taking putative biomarkers into clinically relevant sample types along the disease continuum.

Many of the innovations discussed here fall into the area of new methods for the detection and identification of protein biomarkers. These include the use of cutting-edge technologies in the field of proteomics, including the deployment of laser capture micro-dissection (LCM) to excise diseased and normal tissue from the same CCA lesion; the use of isotopic labeling for relative quantification in such samples; the use of sophisticated hypothesis-driven targeted mass spectrometric analyses for verification; and the combination of these technologies to identify proteins up-regulated in one of the largest collections of tissues derived from confirmed OV-induced CCA patients. In addition there is the use of differential protein expression analysis using the isobaric tag for relative and absolute quantitation (iTRAQ) labeling system and targeted mass spectrometric analyses utilizing multiple-reaction monitoring (MRM), which is unparalleled in its sensitivity, specificity and quantitative abilities.

Similarly, an omics approach could also identify a subset of the differentially expressed miRNAs in CCA samples specifically targeting mRNAs encoding proteins [27–31]. Normal and cancer CCA tissue could be isolated from formalin-fixed, paraffin-embedded tissues. After deparaffinization using xylol and ethanol, miRNA could be isolated and analyzed with a miRNA microarray, interrogating up to 723 human and 76 human viral miRNAs (for examples see [27–31]). Identification of mRNA species targeted by miRNAs will be accomplished computationally using web-based tools such as TargetScan, miRBase and miRanda. It is hypothesized that mRNAs encoding a subset of proteins identified by iTRAQ analysis will serve as targets for miRNAs. Recently, miRNA have been found in abundance in several easily accessible matrices for CCA, including plasmas and feces, making them a potential valuable biomarker target. Moreover, they have also been found for level of liver fibrosis and liver inflammation--precursor stages to *O. viverrini* induced CCA [27, 29].

The development of assays capable of targeting multiple biomarkers will increase the sensitivity and specificity of diagnosis. The need for such an approach is demonstrated by the recent finding that using two potential diagnostic biomarkers for CCA, transthyretin and carbohydrate antigen 19–9, in concert resulted in significant increases in both the sensitivity and selectivity of the detection [32].

Box 4

Technical innovation for Omics studies for biomarkers for CCA

The most advanced proteomic technologies to discover and verify potential biomarkers of CCA:

1. The combination of laser capture micro-dissection with liquid chromatography-tandem mass spectrometry and isotopic labeling using iTRAQ to measure differential protein expression analysis.
2. Multiple Reaction Monitoring (MRM) mass spectrometry to provide targeted detection of a suite of biomarkers simultaneously during the course of a single experiment.
3. Targeting the dysregulation of microRNAs in tissue samples as well as in accessible matrices such as plasma and feces. MicroRNAs also could be used as biomarkers for precursor stages of *O. viverrini* induced CCA such as chronic inflammation and advanced periductal fibrosis [27, 29, 31].
4. Targeting multiple biomarkers increases the potential sensitivity and specificity of the diagnostic method.

A Modest Omics Proposal

Figure 2 shows a potential omics approach to *O. viverrini* induced CCA that would be unique to Khon Kaen Thailand, the epicenter of this lethal cancer. It is comprised two phases and four aims. The two phases consist of a Discovery phase and a Verification phase, with each Aim guided by a unique sampling strategy. Phase 1 constitutes the biomarker discovery stage, where MS is used to identify a suite of up-regulated proteins in frozen resected liver tumor tissue and from CCA cell lines and supernatants. This suite of proteins identified in Aims 1 and 2 would then proceed to Phase 2, i.e. verification. In Aim 3, the presence of candidate biomarkers identified in the discovery phase in plasma samples would be verified in plasma samples paired with the resected liver sections targeted in Aim 1. A number of other non-OV-induced CCA biomarkers that have been identified from the literature could also be verified in plasma samples at this stage (e.g., [32]). The panel of potential biomarkers identified in plasma in Aim 3 could then be taken to a larger more diverse study samples and used to screen plasma of patients with advanced hepatobiliary abnormalities from longitudinal studies of the pathogenesis of OV-induced CCA currently being conducted in Khon Kaen province to verify the biomarkers' potential for early detection of CCA.

Summary

We propose to make a revolutionary leap forward biomarker work for CCA by the deployment of the most advanced omic approaches for discovery and verification of new biomarkers in the epicenter of this lethal cancer, Khon Kaen Thailand. However, this approach requires strategic samples collection and a focus on biobanking as strategic resources for this study of this cancer. Lastly, we emphasize the importance of maintaining cohorts of individuals from an *O. viverrini* endemic region near Khon Kaen to ascertain the value and utility of prospective biomarkers at key steps along the progression from *O. viverrini* infection, through APF to *O. viverrini* -induced CCA.

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Impact of an Omics Approach on Strategically Collected Samples

The *overarching innovation* of such studies is the utilization of a model of human carcinogenesis in which the major risk factor, as well as many of the intermediate stages on the pathway to cancer, have been well- defined. Hence, the presence of biomarkers from the initiation of risk to culmination in CCA could be traced.

Figure 1.

Conceptual framework for the use of biomarkers from OV-induced CCA, with the primary exposure (OV infection) identified and the progression of disease events to CCA tracked in well-defined stages. Figure adapted for OV-induced CCA from Srinivas, Kramer, and Srivastava, *The Lancet*, 2001, Vol 2, p 698 [33].

Figure 2.

Schematic illustration of proposed overall study design as determined by phases of biomarker discovery (phase 1) and biomarker verification (phase 2). The four aims refer to the four different samples types including frozen resected liver, CCA cell lines, plasma from CCA patients (matched to liver sections) and plasma from healthy individual at risk for OV-induced CCA and at various stages of disease progression.