Parasitology International 61 (2012) 173–177

Contents lists available at SciVerse ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Banking on the future: Biobanking for "omics" approaches to biomarker discovery for *Opisthorchis*-induced cholangiocarcinoma in Thailand

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ARTICLE INFO

Available online 9 August 2011

Keywords:
Biomarkers
Cholangiocarcinoma
Liver fibrosis
Opisthorchiasis

ABSTRACT

Cholangiocarcinoma (CCA) – bile duct cancer – is associated with late presentation, poses challenges for diagnosis, and has high mortality. These features t highlight the desperate need for biomarkers than 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.

1. Cholangiocarcinoma from *Opisthorchis viverrini*

At least 750 million people, i.e. 10% of the human population, are at risk of food-borne trematodiases, 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 diet[3]. While the infection can be eliminated by the use of the anthelminthic praziquantel (PZQ), environmental and cultural factors of this region strongly favor routine re-infection [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].

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doi:10.1016/j.parint.2011.06.005

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].

2. 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].

3. 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.

4. 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,