Rev Soc Bras Med Trop 51(2):231-233, March-April, 2018
doi: 10.1590/0037-8682-0180-2017

Short Communication

Negative serology of *Fasciola hepatica* infection in patients with liver cancer in Peru: a preliminary report

***Claudia Machicado*[1],[2], *Stéphane Bertani*[3], *Patricia Herrera-Velit*[1], *Jose Espinoza*[1], *Eloy Ruiz*[4] *and Luis Marcos*[5]**

[1]. Laboratorios de Investigación y Desarrollo, Facultad de Ciencias y Filosofía, Universidad Peruana Cayetano Heredia. Lima, Peru.
[2]. Institute for Biocomputation and Physics of Complex Systems, University of Zaragoza, Zaragoza, Spain.
[3]. Université de Toulouse, IRD, UPS, UMR 152 PHARMA-DEV, Toulouse, France.
[4]. Departamento de Cirugía en Abdomen, Instituto Nacional de Enfermedades Neoplásicas, Lima, Peru.
[5]. Department of Internal Medicine, Division of Infectious Diseases, Department of Microbiology and Genetics, Global Health Institute, Stony Brook University, Stony Brook. New York, USA.

Abstract

Introduction: The etiology of several hepatocellular carcinoma (HCC) cases remains largely unknown. Although *Fasciola hepatica* has been associated with liver fibrosis in Latin America, it has not yet been associated with HCC. This study aimed to determine the existence of specific IgG antibodies against *F. hepatica* in the serum samples of HCC patients. **Methods**: In total, 13 serum samples from 13 HCC patients were screened using Fas2-ELISA. **Results**: Fas2-ELISA demonstrated negative results in all HCC patients included in this study. **Conclusions**: The pre-existence of *F. hepatica* infection in HCC patients needs to be further investigated in epidemiological and experimental studies.

Keywords: Cholangiocarcinoma. Fasciolosis. Fibrosis. Hepatocellular carcinoma. Liver fluke.

More than 20% of cancers in the developing world are associated with an infectious etiology, including hepatocellular carcinoma (HCC) from hepatitis B and C viruses, cholangiocarcinoma (CCA) from the liver fluke *Opisthorchis viverrini*, and gastric adenocarcinoma from the bacteria *Helicobacter pylori*. According to the International Agency for Research on Cancer (IARC), liver and intrahepatic bile duct cancers are the second-most lethal cancers in the world, causing nearly 750,000 deaths in 2012[1]. In South America, liver cancer is the tenth-most fatal cancer. In Peru (a country in South America with the second highest liver cancer incidence rate), a total of 1,767 new cases of liver cancer were diagnosed and 1,716 liver cancer-associated deaths were reported in 2012, making it the fifth-most fatal type of cancer in the country[1]. Given the high mortality and poor survival rates of this malignancy when diagnosed during the late stages, there is an urgent and imminent need to improve our understanding of the biology of this disease in order to facilitate the discovery of new pathways and therapeutic options. The prevalence and etiology of the two types of liver cancer are different. HCC is the most common type of liver cancer in the world and is associated with viral hepatitis infection and aflatoxin consumption. On the other hand, CCA is a rare malignancy that is very uncommon in South America and is associated with fluke infections in Asia. Interestingly, Peru has the highest incidence of primary liver cancer in South America, but such numbers cannot be fully attributed to HBV infections and aflatoxins, which are uncommon[2,3]. Although the prevalence of HCC and CCA in Peru is not independently defined, the existence of both is recognized in the Andean region[4]. Some evidence also indicates that the etiological factors behind a number of reports remain unknown[5]. While obesity and alcohol consumption do not appear to play a role in liver carcinogenesis in Peru, the involvement of other unrecognized etiological agents does seem plausible.

Liver cancer is highly prevalent in the Andean region where some infectious diseases are geographically distributed in similar locations. This is linked to the liver fluke *Fasciola hepatica* that is endemic to the cattle-raising areas of the Andean region[6]. Recent evidence shows that liver cancer in Peru may be associated with an etiological factor different from HBV, given that a large proportion of the disease is neither serologically positive for HBV infection nor identifies an infectious disease as a *bona fide* etiological factor[5,7].

It is well known that the carcinogenic liver fluke *O. viverrini*, a close relative of *F. hepatica*, is a risk factor for CCA[8]. The biology of *O. viverrini*-associated CCA has been described and investigated thoroughly[9]. Moreover, the tissue damage caused by

Corresponding author: Drª Claudia Machicado.
e-mail: claudia.machicado.r@upch.pe
Received 2 May 2017
Accepted 29 September 2017

O. viverrini and a number of metabolites and growth promoting proteins secreted by the liver fluke are recognized carcinogenic promoter factors[10].

Although the liver fluke *O. viverrini* has been associated with cancer in Asia, the role of *F. hepatica* in carcinogenesis remains unknown[11]. In preliminary reports, *F. hepatica* has been shown to cause hepatic damage by inducing fibrosis in the liver parenchyma and by upregulating the genes for liver fibrosis and cirrhosis in infected animals[12]. As liver fibrosis is widely known to be a major risk factor for liver cancer, we postulate that a history of *Fasciola* infection may be associated with liver cancer in Peru.

In order to investigate our hypothesis, we assessed the existence of circulating antibodies against the *F. hepatica* Fas2 antigen in patients diagnosed with liver cancer by conducting Fas2-ELISA, an accurate serological test based on the detection of circulating immunoglobulin G (IgG) antibodies elicited in infected individuals against Fas2. A total of 13 serum samples were analyzed. These biospecimens were previously obtained from an HCC study conducted at the Instituto Nacional de Enfermedades Neoplásicas (INEN). All the samples corresponded to patients diagnosed with liver cancer at INEN between 2014 and 2015. Written informed consent was obtained from each patient before sample collection. Demographic data was also recorded, which included the sex, age, tumor type, place of current residency, and place of birth. Serum samples were preserved at -80°C under laboratory conditions at INEN until delivery to the Laboratorios de Investigación (LID) at the Universidad Peruana Cayetano Heredia (UPCH) for testing.

The Fas2-ELISA test was conducted at UPCH as described previously[13]. Briefly, Fas2 (300ng/well) was bound to microtiter plates Immulon® 4HBX (Dynex Technologies, Inc., Chantilly, VA, USA) via incubation for 16 hours at 4°C. The plates were washed five times with Phosphate Buffered Saline (PBS) containing 0.05% Tween-20 (PBST) and then incubated in 2% Bovine Serum Albumin (BSA) in PBST (PBSTB) for 1 hour at 37°C. Serum (100µL) that was previously diluted (1/500 in PBSTB) was added to the well and incubated for 1 hour at 37°C. The plates were washed five times with PBST. Next, goat anti-human IgG conjugated to horseradish peroxidase (100µL) was diluted to 1/2000 in PBSTB, added to each well, and then incubated for 1 hour at 37°C. A color reaction developed when 3,3',5,5'-tetramethylbenzidine (TMB) was incorporated into the reaction, which was stopped by adding 50mL of 2M H_2SO_4. The optical density was measured at 450nm in a microplate reader (Benchmark Bio-Rad). Negative and positive controls were used. The results obtained using Fas2-ELISA were analyzed considering the demographic and relevant clinical data of each patient.

Ethical considerations

This study was approved by the Institutional Ethics Committee of UPCH under the approval ID 650516 and by the Human Subjects Committee of The Instituto de Enfermedades Neoplasicas (INEN) under the approval ID 113-2014-Comit Institucional de Etica (CIE)/INEN.

The main characteristics of the study patient group are shown in **Table 1**. The mean age of the 13 patients was 41 years (range, 22-84). Four women and 9 men were included in the study. Eight (61.5%) patients were born in the Andean region of Peru (endemic areas for *Fasciola*), whereas the other 5 (38.5%) patients were born in non-endemic areas for *Fasciola*. Twelve patients had HCC as the primary tumor and 1 patient had a metastatic CCA located in the liver.

The Fas2-ELISA test was performed on the 13 study samples along with a positive and negative control. The reference values were established as optical density (OD) ≥ 0.2 for positive results and OD < 0.2 for negative results. The OD of the 13 study samples ranged from 0,019 to 0,156, demonstrating negative Fas2-ELISA results for all the analyzed samples.

Increasing evidence suggests the existence of etiological agents other than HBV and aflatoxins with respect to HCC incidence in Peru[14]. Given the close phylogenetic relationship of the carcinogenic liver fluke *O. viverrini* with *F. hepatica*, we hypothesized that the latter may be involved in idiopathic cancer in Peru. To explore our hypothesis, we assessed the existence of *F. hepatica* in a group of 13 patients diagnosed with liver cancer. The validated and accurate Fas2-ELISA test (a currently used routine serological test for *Fasciola* infection in Peru) was used to examine the existence of the circulating *F. hepatica* anti-Fas2 ligand. Our results showed that *F. hepatica* was absent in the serum samples analyzed in this study. Although the study showed negative results, exposure and previous fascioliasis cannot be discarded as valid factors. *F. hepatica*-infected animals usually increase the amount of circulating and highly-specific anti-Fas2 IgG antibodies, thus allowing the disease to be detected as early as 10 days after infection using Fas2-ELISA[15]. However, anti-Fas2 IgGs are short-lived and have an estimated half-life of less than 6 months[13], which does not allow for the accurate detection of *F. hepatica* using Fas2-ELISA 6 months post-infection. Therefore, our results need to be interpreted with caution.

In conclusion, none of the analyzed samples in this study showed the presence of antibodies against *F. hepatica* at the time of the test, suggesting that either the HCC patients were not recently infected or that they probably acquired the infection up to 6 months before sample collection. Whether fascioliasis is a condition that promotes liver cancer as a long-term effect is something that needs to be evaluated via epidemiological studies and experimental assays.

Acknowledgements

The authors thank the financial assistance given to the work.

Conflict of interest

The authors declare that there is no conflict of interest.

Financial support

This work was supported by the Third Cancer Plan of the French National Alliance for Life Sciences and Health (ENV201408) and by the Young Research Teams Associated with IRD Program (JEAI-INCAncer).

TABLE 1: Summary of study patient group characteristics.

Patient number	Age	Sex	Tumor type	Living place	Birth place	OD value* (Fas2-ELISA)	Fas2-ELISA result
1	24	M	HCC	Lambayeque	Lambayeque	0.047	Negative
2	41	M	HCC	Lima	Lima	0.152	Negative
3	37	F	HCC	Lima	Lima	0.064	Negative
4	18	M	HCC	Lima	Junín	0.066	Negative
5	33	M	HCC	Lima	Lima	0.021	Negative
6	30	M	HCC	Huancavelica	Huancavelica	0.019	Negative
7	27	M	HCC	Junín	Junín	0.055	Negative
8	61	M	HCC	Cusco	Cusco	0.052	Negative
9	22	F	HCC	Ayacucho	Ayacucho	0.098	Negative
10	58	M	HCC	Junín	Junín	0.09	Negative
11	81	M	HCC	Lima	Huánuco	0.045	Negative
12	84	F	CCA with liver metastases	Lima	Lima	0.023	Negative
13	23	M	HCC	Huánuco	Huánuco	0.04	Negative

OD: optical density; ***Fas2-ELISA:*** enzyme-linked immunosorbent assay; **M:** male; **F:** female; **HCC:** hepatocellular carcinoma; **CCA:** cholangiocarcinoma. *OD measured at 450nm.

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