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ORIGINAL ARTICLE

Role of microRNAs in host defense against *Echinococcus granulosus* infection: a preliminary assessment

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Abstract

Cystic echinococcosis (CE) is a neglected helminthic zoonosis caused by the larval stage of the tapeworm *Echinococcus granulosus s.l.* MicroRNAs (miRNAs) are regulators of gene expression that have been linked with the pathogenesis of several human diseases, but little exists in the available literature about miRNAs in CE. Here, we investigate the expression profiles of 84 microRNAs relevant to the function of lymphocytes and other immune cells during CE infection in the peripheral blood of patients with cysts in active and inactive stages. We applied the microRNA PCR array technology to blood samples from 20 patients with a single hepatic CE cyst in either the active (CE3b) or inactive (CE4–CE5) stage. Our results show a significant upregulation of eight miRNAs (let-7g-5p, let-7a-5p, miR- 26a-5p, miR- 26b-5p, miR- 195-5p, miR- 16-5p, miR- 30c-5p, and miR- 223-3p) in patients with active cysts compared to those with inactive cysts. The high expression of these miRNAs in patients with active cysts suggests their role in a specific host immune response against the infection. Further work in this direction may help shed light on the pathogenesis of human CE.

Keywords MicroRNA · Cystic echinococcosis · *Echinococcus granulosus* · Hydatidosis · Zoonosis · Neglected disease

Introduction

Cystic echinococcosis (CE) is a chronic helminthic disease caused by the larval stage of the tapeworm *Echinococcus granulosus* sensu lato species complex. The life cycle of this parasite involves two hosts: dogs as definitive hosts and livestock (particularly sheep) as intermediate hosts, respectively. Humans act as accidental intermediate hosts. *E. granulosus* is distributed worldwide, with an estimated 1.2 million human cases [1] but CE is still a neglected disease [2]. In humans, the larval stage of the parasite develops as fluid-filled cysts that enlarge concentrically, up to a diameter of 20 cm or more, and may survive for years or decades. These cysts are mainly localized in the liver (70%) and the lungs (20%) but may develop in any organ or tissue. The diagnosis and follow-up of CE is based on imaging. The WHO Informal Working Group on Echinococcosis (WHO-IWGE) developed a standardized classification of CE cysts based on ultrasound morphology [3], which also indicates the activity of the cyst and guides the clinical management of the patient [4]. Currently, the other diagnostic tool available for the diagnosis of CE is serology. However, serological tests suffer from many limitations, including false positives, false negatives, and the scarce utility in the follow-up of patients due to their inability to distinguish between cysts that are definitely inactive and cysts with a potential for reactivation. Serology results are also influenced by several variables, including the cyst size, stage, and number [5]. As such, new diagnostic and prognostic tools are much needed in the management of CE.

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The mechanisms underlying the cohabitation of host and parasite are still unclear. Experimental studies in the past 30 years have tried to explain the strategies that parasites enact to survive within their hosts. Recent studies have shown that microRNAs (miRNAs) have an active role in the host–pathogen interaction and host immune responses to microorganisms [6]. MiRNAs are small (19–24 nucleotide), non-coding RNAs that regulate gene expression post-transcriptionally by inhibiting protein translation or destabilizing target transcripts [7]. MiRNAs play an important role in hematopoiesis, immune response, and inflammation, and have emerged in recent years as important regulators of both innate and adaptive immune responses in a variety of murine model systems [8–10]. MiRNAs are also involved in the pathogenesis of several human diseases including malignancies and diseases related to the immune response [9, 10].

Recent studies showed that circulating miRNAs of both parasite and host origin can be detected in blood or fluids of humans and animals with helminth infections [11]. For this reason, they are explored as potentially diagnostic biomarkers for the early detection of parasite infection or related diseases. For example, human miR-192 has a potential utility as a non-invasive prognostic indicator for liver fluke-associated cholangiocarcinoma [12]. With respect to *Echinococcus* species miRNAs, they have recently been described providing a possibility of understanding their roles in host–parasite interaction, and their future potential use as diagnostic targets [13, 14].

Microarray technology is a valuable tool for the identification and characterization of gene expression profiles, due to its ability to analyze the differentially expressed genes of a whole genome in a single experiment. The aim of this study was to determine if human miRNAs related to immunity are differentially expressed during *E. granulosus* infection.

Materials and methods

Sample collection

Twenty venous blood samples from 20 patients with a single hepatic CE cyst were included in the study. All patients were diagnosed by ultrasound (US). Cysts were classified according to the WHO-IWGE classification [3] and were tested for routine diagnostic purposes in our diagnostic parasitology lab at the San Matteo Hospital Foundation, Pavia, Italy, using ELISA (RIDASCREEN® Echinococcus IgG, R-Biopharm, Darmstadt, Germany) and IHA (Cellognost® Echinococcosis IHA, Siemens Healthcare Diagnostics, Marburg, Germany) tests, as per manufacturer instructions. Cysts without pathognomonic signs of CE on US were etiologically confirmed based on positive results on at least one serology test confirmed by the presence of specific bands on Western blot (ECHINOCOCCUS® Western blot IgG LDBIO Diagnostics, Lyon, France). Samples were collected at our clinic, stored at − 80 °C until use, and divided into two groups. Group 1 included 10 blood samples from patients with active cysts (CE3b) and group 2 included 10 blood samples from patients with inactive cysts (CE4 and CE5) that reached inactivation spontaneously.

Ethics statement

All patients signed an informed consent form for storage and scientific use of the leftover serum at the moment of blood sampling for routine serology. This retrospective study was performed according to the guidelines of Institutional Review Board of San Matteo Hospital Foundation, Pavia, Italy, on the use of biological specimens for scientific purposes in keeping with Italian Law (art.13 D.L gs 196/2003). All procedures performed in studies involving human participants were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

RNA extraction and reverse transcription

Total RNA was extracted from whole blood samples using TRIzol LS Reagent (Ambion; Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions, and quantified using a ND-100 Spectrophotometer (NanoDrop™ Technologies, Wilmington, DE, USA).

For each sample, 250 ng of total RNA was retrotranscribed to cDNA using miScript II RT Kit (Qiagen, Hilden, Germany), as per manufacturer's protocol. Briefly, mature miRNAs were polyadenylated by poly(A) polymerase and converted into cDNA by reverse transcriptase with oligo-dT priming. The cDNA was then used for real-time PCR quantification of mature miRNA expression using the miScript miRNA PCR assay (Qiagen, Hilden, Germany).

MicroRNA PCR array

Quantification of miRNAs in blood was carried out using Human Immunopathology miScript miRNA PCR array (Qiagen, Hilden, Germany). The Human Immunopathology miScript miRNA PCR array profiles the expression of 84 miRNAs differentially expressed during immune responses. All reactions were carried out according to the manufacturer's protocols and recommendations using the IQ 5 Real-time PCR (BioRad, Hercules, CA, USA) detection system.

Statistical analysis

The relative expressions of miRNAs were calculated using the 2-ΔCt method. The differences of miRNA levels between patients with inactive and active cysts were evaluated using a Mann-Whitney non-parametric test. All analyses were

performed using Stata v 15.2 (StataCorp, USA) software. A $p < 0.01$ was considered as significant.

Results

Sample RNA quality control

RNA concentration and purity were determined by NanoDrop ND-1000 spectrophotometer. The absorbance 260/280 ratio was ≥ 2 in all the samples. In addition, the integrity of RNA of several samples was determined by 1% agarose gel electrophoresis. The concentration of all the RNA samples was higher than 25 ng/μl, and the purity and integrity were suitable for microarray experiments in the present study.

miRNA profiling by miRNA PCR array analysis

To screen the expression of miRNAs in patients with different cyst stages, miRNA profiling by miRNA PCR array was performed on serum samples from 20 patients with CE with a single hepatic cyst. The microarray analysis indicated an upregulation in the expression of eight miRNAs (let-7g-5p, miR-26a-5p, miR- 195-5p, let-7a-5p, miR- 16-5p, miR- 26b-5p, miR- 30c-5p, and miR- 223-3p) in patients with active cysts compared with patients with inactive cysts. In more detail, let-7g-5p, let-7a-5p, miR- 26a-5p, and miR- 26b-5p showed an upregulation of 15.3-, 9.7-, 11.2-, and 8.4-fold, respectively (all $p < 0.01$; Fig. 1a); miR 195-5p and miR 16-5p, two members of the miR-15 family, showed an upregulation of 5.7- and 12.3-fold, respectively (all $p < 0.01$; Fig. 1b) and miR 30c-5p and miR 223-3p showed an upregulation of 6.3- and 6.2-fold, respectively (all $p < 0.01$; Fig. 1c).

Discussion

CE is a chronic, complex, and neglected disease with a worldwide distribution, especially in livestock-raising areas [15]. The spectrum of clinical manifestations ranges from asymptomatic to even fatal. The pathogenesis of CE is still unclear. The natural history of the cysts is not completely known; they appear to pass through different stages, from active to inactive forms [16], as classified by the WHO-IWGE [3], and to date the interplay between host and parasite remains largely unknown.

In the last years, many research groups focused their attention on the relationships between miRNAs and immunity in both human and animals and on the changes occurring in the global miRNA expression patterns upon infection [17, 18]. However, extremely little is known in this field regarding miRNAs in parasitic infections in the human host [11].

In this study, we analyzed the expression profiles of 84 miRNAs involved in the regulation of the immune response in whole blood samples from 20 patients with CE in active and inactive stages using a miRNA PCR array. Our results show a significant upregulation of eight miRNAs (let-7g-5p, let-7a-5p, miR- 26a-5p, miR- 26b-5p, miR- 195-5p, miR- 16-5p, miR- 30c-5p, and miR- 223-3p) in patients with active cysts compared to those with inactive cysts (Fig. 1).

These miRNAs were already known to be involved in human diseases and widely studied in animal models. Let-7 family and miR-26 are known to have a direct role in immune responses such as proliferation and activation of macrophages, inflammation, apoptosis, and/or oxidative damage [19]. In particular, the let-7 family members have been shown to target interleukin (IL)-13, IL-10, and IL-6 in in vivo and in vitro models; however, results are still contrasting [20], while miR-26a5p and miR-26b-5p are frequently downregulated in various types of cancer, suggesting that these miRNAs function as tumor suppressors by targeting multiple oncogenes [21]. MiR-26a also increases the expression of type I interferon, a signaling protein released by host cells in response to the presence of several pathogens, such as bacteria, parasites, and viruses, and also tumor cells [22], while miR-26b modulates the NF-jB pathway in alveolar macrophages by regulating PTEN [23]. MiR 195-5p and miR 16-5p belong to the miR-15 family; they are implicated in promoting apoptosis in a variety of cell types including immune cells, epithelial cells,

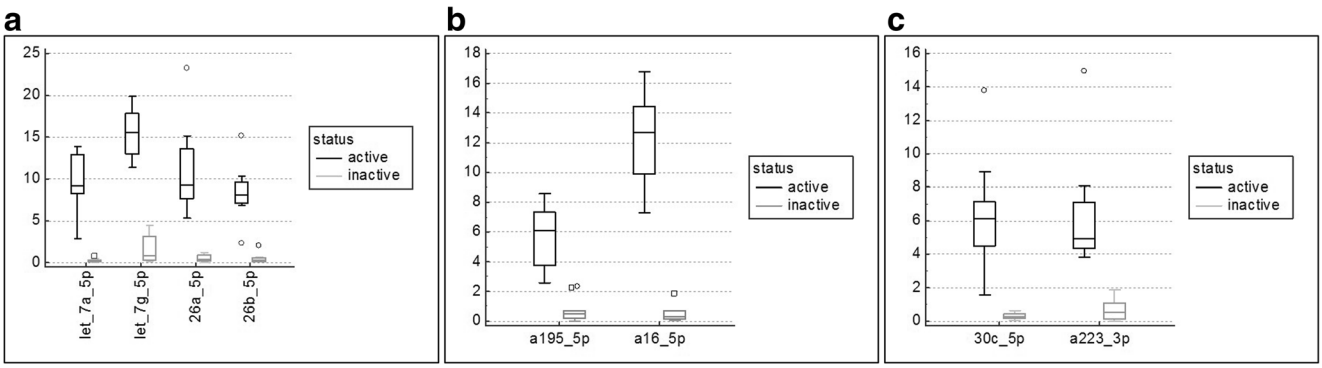

Fig. 1 **a**, **b**, and **c** Expression levels of circulating miRNA in patients with active (black) and inactive (gray) cyst associated with the regulation of genes involved in the control of molecules connected with the immune system and signaling and transport of proteins

and other tissue cells, that have been found in both animal and human studies [24–26]. MiR-30 and miR-223 have a key role in the regulation of the innate immunity and in the type I interferon signaling and other cytokines, although their relative up- and downregulation are not consistent among studies [27, 28]. Both those miRNAs are also known to function as a tumor suppressor involved in many types of cancers [29, 30].

Despite the small sample size analyzed, our study demonstrates a statistically significant upregulation of eight miRNAs (let-7g-5p, let-7a-5p, miR- 26a-5p, miR- 26b-5p, miR- 195-5p, miR- 16-5p, miR- 30c-5p, and miR- 223-3p) associated with the presence of active cysts and suggesting involvement of host miRNAs in the human–parasite interplay with the *E. granulosus* metacestode. These results add to the data obtained by Guo et al. and Jiang et al., who observed a dysregulation of the expression of several host miRNAs in *E. multilocularis*-infected mouse sera and in infected CE-resistant and CE-nonresistant sheep gut during peroral *E. granulosus* infection, respectively [31, 32]. The immune response to established hydatid cysts is poorly known; as summarized in a recent review [33], it is evident that immune modulatory mechanisms are in place, allowing the persistence of the established metacestode in the intermediate host for many years. In the intermediate host, cysts pass through different stages, in some occasions resulting in the spontaneous inactivation of the cyst. Although a different cell infiltrate can be observed surrounding intact and regressive cysts, it is unclear whether this is the cause or the effect of cyst structural changes. As a whole, however, current data from mouse models point to a possible damaging effect of a mixed Th1/Th2 immune response. Given the discrepancies in the literature on the correlation between different miRNAs and immune effector mechanisms, it is not possible, at this stage, to attempt even speculations on the upregulation of miRNA found in our study in relation to the cyst activity and a defined pro- or anti-inflammatory environment. These preliminary data, however, open the way to further studies on whether these miRNAs are related to *E. granulosus* infection and, if so, the pathogenetic role of miRNAs in this complex disease.

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Conflict of interest The authors declare that they have no conflict of interest.

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