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MicroRNA expression profile in patients with cystic echinococcosis and identification of possible cellular pathways

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Research Paper

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Abstract

Cystic echinococcosis (CE) is a neglected tropical disease, caused by metacestode (larval) form of the *Echinococcus granulosus* sensu lato (sl) in humans. MicroRNAs (miRNAs) are small, stable, tissue-specific RNA molecules encoded by the genome that are not translated into proteins. Circulating miRNA expression profiles vary in health and disease. The aim of this study is to determine the altered cellular pathways in CE by comparing the miRNA profiles of controls and CE patients with active or inactive cysts. Following abdominal ultrasonography (US) examination, 20 patients diagnosed with active CE (CE1, CE2, CE3a and CE3b) or inactive CE (CE4 and CE5) and three healthy controls were included in the study. The expression profiles of 372 biologically relevant human miRNAs were investigated in serum samples from CE patients and healthy controls with miScript miRNA HC PCR Array. Compared with the control group, expression of 6 miRNAs (hsa-miR-4659a-5p, hsa-miR-4518, hsa-miR-3977, hsa-miR-4692, hsa-miR-181b-3p, hsa-miR-4491) and one miRNA (hsa-miR-4687-5p) were found to be downregulated in CE patients with active and inactive cysts, respectively ($p < 0.05$). For downregulated miRNAs in this study, predicted targets were found to be associated mainly with cell proliferation, apoptosis, cell-cell interactions and cell cycle regulation. Further studies in this direction may elucidate the pathogenesis of human CE and the relationship between CE and other pathologies.

Introduction

Cystic echinococcosis (CE) is a neglected disease caused by metacestode (larval) form of the *Echinococcus granulosus* sensu lato (sl) in humans. CE is mostly endemic in rural areas of Australia, Asia, South America and Mediterranean countries (Deplazes *et al.*, 2017). Since it is mostly asymptomatic, definite prevalence of the disease is still unknown. However, the incidence of human CE is reported based on hospital records (Altintas, 2008). As reported in a recent study, CE prevalence is estimated to be approximately 0.61% in Turkey (Tamarozzi *et al.*, 2018). Although CE is mostly associated with a wide spectrum of symptoms, there is no specific finding. The hydatid cysts are mostly located in the liver and lung, with rates of about 70% and 25%, respectively (Akhan *et al.*, 1996; Brunetti *et al.*, 2018). Imaging techniques such as ultrasonography (US), computed tomography (CT) and magnetic resonance imaging (MRI) are extensively used for diagnosis of CE. Serological tests have complementary role to imaging modalities in diagnosis. The World Health Organization-Informal Working Group on Echinococcosis (WHO-IWGE) has published a widely accepted classification of liver hydatid cyst based on the activity of the disease. Based on this classification, hydatid cysts are divided into three clinical groups as active (CE1 and CE2), transitional (CE3a/CE3b) and inactive (CE4 and CE5) (Brunetti *et al.*, 2010; Kern *et al.*, 2017). Although imaging techniques generally provide sufficient data for the diagnosis of CE, serological tests are needed in some cases. Unfortunately, there are considerable drawbacks in sensitivity/specificity (Se/Sp) and prognostic value of serological tests (Akhan & Ozmen, 1999; Manzano-Román *et al.*, 2015). While several studies are reported that cyst characteristics affected CE serology, there is still limited data available on underlying mechanisms of the host during CE development and progression.

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules, that have function in RNA silencing and post-transcriptional regulation of gene expression. Some miRNA families are predominantly expressed in certain tissues while some others are specific to certain biological processes (Negrini *et al.*, 2009; Li *et al.*, 2010). Unique circulating miRNA expression profiles have been demonstrated for various types of diseases. Mammalian miRNAs are

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Table 1. Characteristics of patients.

Number	Gender	Age	Cyst number	Cyst type	Cyst location in liver	Cyst Size (cm)	Cyst volume (cm^3)
1	Female	24	1	CE1	Right lobe	5–10	110.26
2	Female	37	1	CE1	Left lobe	5–10	168.48
3	Female	25	1	CE1	Left lobe	>10	884
4	Female	21	1	CE1	Right lobe	>10	892.32
5	Female	46	1	CE1	Left lobe	5–10	278.46
6	Female	54	1	CE2	Right lobe	>10	280.28
7	Male	42	1	CE2	Right lobe	5–10	219.37
8	Female	30	1	CE2	Left lobe	<5	65
9	Male	7	1	CE2	Left lobe	5–10	84.5
10	Female	18	2	CE3b	Right lobe	>10	224.64
11	Male	26	1	CE3a	Left lobe	<5	62.4
12	Male	30	1	CE3b	Right lobe	5–10	203.84
13	Male	64	1	CE4	Right lobe	5–10	139.94
14	Female	33	2	CE4	Right lobe	5–10	178.36
15	Female	41	1	CE4	Right lobe	5–10	219.37
16	Male	31	2	CE4	Right lobe	<5	56.62
17	Female	52	1	CE4	Right lobe	>10	484.38
18	Male	69	1	CE5	Right lobe	<5	53.76
19	Female	60	1	CE5	Right lobe	<5	40.95
20	Female	44	1	CE5	Left lobe	5–10	374.86

known to be stable in extracellular fluids such as plasma, serum, urine, saliva and semen (Mitchell *et al.*, 2008; Olivieri *et al.*, 2017). With the discovery of the disease-specific miRNAs in the blood of patients with cancer, metabolic disorders or viral infections, miRNA expression profiles have been widely studied particularly in infectious diseases that are difficult to diagnose and follow-up. (Tritten *et al.*, 2014). Besides, miRNAs have a crucial role in the regulation of host–pathogen relation due to their function as post-transcriptional mechanism regulators (Cai *et al.*, 2016).

The aims of this study are (1) to determine the alterations in miRNA expression profiles of patients with active and inactive cysts compared with healthy controls and (2) to identify altered cellular pathways in CE patients.

Materials and methods

Ethics statement

This study was approved by the Institutional Ethical Committee of the Faculty of Medicine (GO 17/711-16).

Sample collection, RNA extraction and cDNA synthesis

Twenty confirmed CE patients with 23 CE cyst (13 female and seven male) and three healthy controls (one female and two male, mean age ±40, without any underlying chronic and infectious disease) were included in this study. During US examination, the WHO-IWGE classification was used to evaluate the cases (Brunetti *et al.*, 2010; Kern *et al.*, 2017). Total RNA extraction was performed from serum of the patients/controls by miRNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Quality and purity of the RNA was verified by a NanoDrop 2000c instrument (ThermoScientific). For cDNA synthesis, miScript II RT system (Qiagen) were used according to manufacturer's recommendations.

miRNA expression profiles and pathway analyses

miScript SYBR Green PCR Kit and miScript miRNA HC PCR Arrays (with a LightCycler 480 instrument II (Roche, Germany)) were used for the detection and quantification of miRNAs in serum. The miScript miRNA HC PCR Array provides expression profiles of 372 pathway/disease/functionally related mature miRNAs. Data analysis was performed using an online Geneglobe data analysis centre (https://geneglobe.qiagen.com/no/analyze/), which uses the comparative CT (ΔΔCT) method for relative quantification and indicates fold change calculations. $p < 0.05$ was considered as statistically significant.

For miRNA target prediction miRDB (http://mirdb.org/), Targetscan (http://www.targetscan.org/vert_72/), and DIANA Tools (http://diana.imis.athena-innovation.gr/DianaTools/index.php) were used (Friedman *et al.*, 2009; Paraskevopoulou *et al.*, 2013; Liu & Wang, 2019; Chen & Wang, 2020).

Results

Demographics of patients and clinical characteristics

The majority of the cases were female (65%, 13/20). Mean age of the patients were 37.7 (range 7–69 years).

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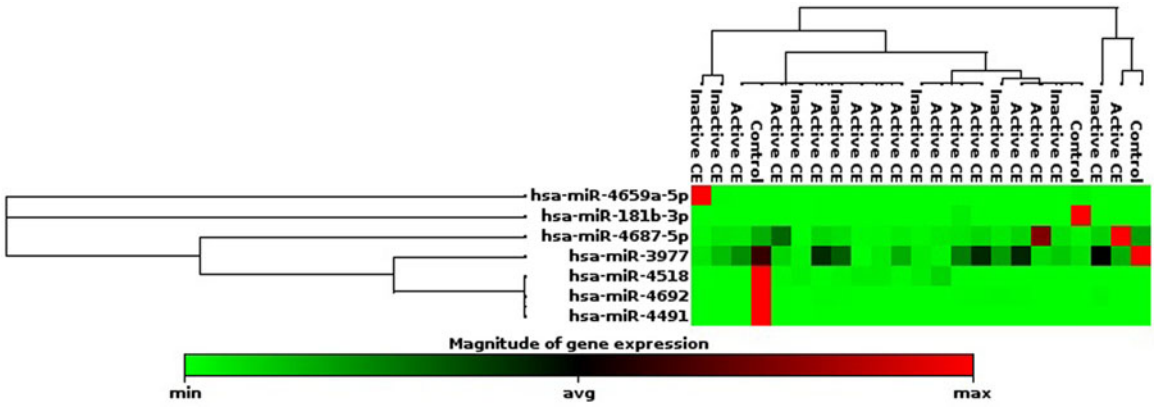

Fig. 1. Heatmap of miRNA expressions among CE patients and healthy control groups. The map is produced by using the online GeneGlobe Data Analysis Center (https://geneglobe.qiagen.com/no/analyze/).

Five of the 23 hydatid cysts were identified as CE1, four as CE2, one as CE3a, three as CE3b, seven as CE4 and three as CE5. Among 20 patients, 17 had a single cyst, the rest harboured two hydatid cysts. None of the patients had more than one cyst type. The mean size and volume of cysts were recorded as 7.4 cm and 251 cm^3, respectively. All cysts were located in the liver (14/23 right lobe, 9/23 left lobe) (table 1). All the serum samples were positive for hydatidosis either by enzyme-linked immunosorbent assay (Hydatidosis IgG ELISA, Vircell SL, Granada, Spain) or indirect hemagglutination assay (Hydatidose, FUMOUZE Laboratories, France).

miRNA expression profiles

In patients with active cysts, a total of 6 miRNA (hsa-miR-4659a-5p, hsa-miR-4518, hsa-miR-3977, hsa-miR-4692, hsa-miR-181b-3p, hsa-miR-4491) and in inactive CE patients only one miRNA (hsa-miR-4687-5p) expression was found to be downregulated by at least twofold ($p < 0.05$) compared with healthy controls. Relative expression profiles of these miRNAs in active and inactive patients are presented in figs 1 and 2.

Pathway analysis

Downregulated miRNAs in active CE patients mainly regulate the expressions of cancer related oncogenes and tumour suppressor genes through cell proliferation, cell cycle, cell–cell interaction, transport systems, DNA repair and translational regulation. Additionally, these miRNAs can also play a role in neuronal growth process, function, differentiation and expression of immunoglobulin superfamilies.

Downregulated miRNA in inactive CE patients mainly regulates various cellular processes, including cell cycle progression, signal transduction, apoptosis, and gene regulation.

Discussion

Since the correlation between the expression of miRNAs in circulation and various pathologies has been determined, miRNAs are thought to be promising diagnostic biomarkers (Schwarzenbach *et al.*, 2014). Although many studies focused on miRNA expression profiles in parasitic infections, there are only two studies conducted on the miRNA-based analysis of CE patients. In the study conducted by Mariconti *et al.*, the expression profile of immune-related miRNAs was reported to be altered between active and inactive CE patients (Mariconti *et al.*, 2019). According to their findings, six miRNAs in active CE patients were found to be upregulated compared with inactive CE patients. miRNAs detected in their study, have role in various immune-related processes such as proliferation/activation of macrophages, inflammation, apoptosis and/or oxidative damage, the regulation of the innate immunity, the type I interferon signalling and tumour suppression in many types of cancers (Mariconti *et al.*, 2019). In the other study, parasite-derived miRNAs, egr-miR71 and egr-let 7, which were detected in circulation of CE patients found to be decreased after the removal of the cyst. These miRNAs are considered promising biomarkers for CE; however, the study does not provide any information about the pathways affected in the host in presence of CE (Alizadeh *et al.*, 2020).

According to our results, compared with control group downregulation in six miRNAs and one miRNA expressions were determined in active and inactive CE patients, respectively. These miRNAs were already known to be mainly involved in cell proliferation, apoptosis, cell–cell interactions and cell cycle. Current knowledge has suggested that miRNAs can play antitumoral or oncogenic roles in different cancer types. It is known that hsa-miR-4659a-5p, hsa-miR-4518, hsa-miR-181b-3p and hsa-miR-4687-5p, which were determined to be downregulated in this study, have a role in cancer development. In particular, an association between hsa-miR-4659a-5p and advanced breast cancer has been reported (Tabatabian *et al.*, 2020). In a recent study, miR-4518 expression was found to be downregulated significantly in glioma tissues (Lu *et al.*, 2018). miR-181b-3p was found to induce epithelial–mesenchymal transition which is crucial for increased invasion and metastasis during cancer progression in MCF7 breast cancer cells (Yoo *et al.*, 2016). Additionally, miR-181b-3p was recently found as one of the miRNAs with potential in discriminating neck lymph node metastasis, suggesting that this miRNA has a potential as a prognostic biomarker (Liu *et al.*, 2020). Lastly, miR-4687-5p was found to be upregulated in lung adenocarcinoma metastasis and considered to be among potential miRNA biomarkers for small cell lung cancer (Xu *et al.*, 2020).

Some parasitic infections are well-known risk factors for various types of cancer in mammalian hosts. On the other hand, reports on the anticancer effects of parasitic organisms are limited. However, there are conflicting reports on the effect of *E. granulosus* on cancer risk. A retrospective study on CE

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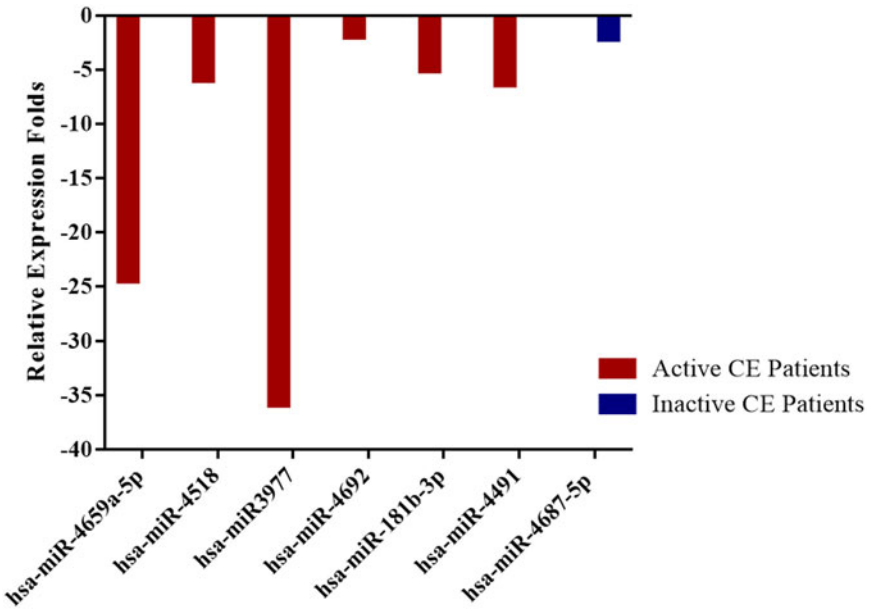

Fig. 2. Compared to control group downregulated miRNAs in active and inactive CE patients with a significant relative expression fold change ($p < 0.05$).

suggested a negative correlation between CE and solid tumours (Akgül *et al.*, 2003). In contrast, Oikonomopoulou *et al.* proposed that infection by *E. granulosus* may increase the risk of cancer (Oikonomopoulou *et al.*, 2016). Relationship between CE and cancer has not been clearly defined yet. Many studies also suggest a protective effect of *E. granulosus* and its products against cancer development. However, molecular mechanisms still need to be clarified (Guan *et al.*, 2019).

To date, several mechanisms have been proposed to explain CE related anticancer effects, including parasite molecules and activation of host immune response. Ranasinghe *et al.* proposed that Kunitz-type protease inhibitor EgKI-1 which is highly expressed by the oncosphere of *E. granulosus* could disrupt the regular cell cycle and induce apoptosis in cancer cells (Ranasinghe *et al.*, 2015, 2018).

In this study, most of the downregulated miRNAs in CE patients were found to be associated with cell proliferation, apoptosis, cell–cell interactions and cell cycle. Downregulation of these miRNAs due to the presence of the CE cyst possibly play a regulatory role in the anti-cancer effect.

This research has particular limitations. The patients in this study were limited to liver CE Thus, further research including CE patients with other organ involvement like lungs, kidneys, etc., is needed to verify whether these miRNAs play an essential role in *E. granulosus* infection.

In conclusion, we showed that profiles of miRNA expression vary in active and inactive CE cysts of the liver. According to pathway analysis based on alterations in miRNA profiles, targets were predicted to involve mainly cell proliferation, apoptosis, cell–cell interactions and cell cycle control. miRNAs downregulated in CE should be considered as promising molecules in the mechanism based on the relation between cancer development and CE. Further studies based on host miRNAs are needed to confirm the affected genes in the presence of CE and to enlighten underlying molecular mechanisms of the relationship between CE and cancer.

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Conflicts of interest. None.

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