



IF: 1.634

Asian Pacific Journal of Tropical Medicine

journal homepage: www.apjtm.org



doi: 10.4103/1995-7645.248341

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Can miRNA712_3p be a promising biomarker for early diagnosis of toxoplasmosis?

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ARTICLE INFO

Article history:

Received 5 July 2018

Received in revised form 10 December 2018

Accepted 19 December 2018

Available online 26 December 2018

Keywords:

Toxoplasma gondii

microRNAs

miRNA712_3p

Biomarker

Real-time PCR

ABSTRACT

Objective: To assess the role of miRNA712_3p as a specific biomarker in early detection of toxoplasmosis in plasma of mice acutely infected with *Toxoplasma gondii*. **Methods:** Real-time PCR was used to measure the level of miRNA712_3p in plasma of infected mice. Immune-competent and immune-suppressed mice were examined, three and five days post-infection. **Results:** Results revealed significant up-regulation of plasma miRNA712_3p in both immune-competent and immune-compromised groups in comparison to the control non-infected group. Additionally, an increase in the level of miRNA712_3p was noticed correspondently in the parasite density detected in liver impression smears. **Conclusions:** miRNA712_3p can be used as a novel biomarker for the detection of *Toxoplasma gondii* infection in both immune-competent and immune-compromised host.

1. Introduction

Toxoplasma gondii (*T. gondii*) is a highly successful obligatory intracellular protozoan. It is considered as one of the most prevalent parasites in the world with a wide range of hosts, including humans, causing toxoplasmosis. Nearly, one-third of the total human population is estimated to be seropositive[1]. People acquire the infection by three principal routes: foodborne, zoonotic from animals and congenitally from infected mother to her fetus. Organ transplant, blood transfusion and laboratory accidental infections are less common routes of human toxoplasmosis[2].

Toxoplasmosis has a wide spectrum of clinical picture ranging from life-threatening condition to long-term chronic asymptomatic infection. The life cycle of *T. gondii* is unique, as the parasite is capable of indefinite proliferation in its hosts with either sexual or

asexual cycles. The sexual cycle occurs only in a feline host (cat), while the asexual cycle can occur in any warm-blooded animal. The parasite is capable of invading any nucleated cell within only 20 s, with preference to muscular and neural tissues in chronic condition[3].

Although infection in immune-competent adults is typically subclinical, it could persist for life. On the other hand, severe fatal consequences may occur in case of congenitally infected infants or in immune-compromised hosts[4,5]. Treatment of pregnant women could reduce the incidence of sequels in congenitally infected infants[6]. Moreover, it is needless to mention that early detection of

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How to cite this article: Mogahed NMFH, Khedr SI, Ghazala RA, Masoud IM. Can miRNA712_3p be a promising biomarker for early diagnosis of toxoplasmosis? Asian Pac J Trop Med 2018; 11(12): 688-692.

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T. gondii infection can be considered as a key measure in reducing toxoplasmosis-related health problems in both immune-competent and immune-compromised individuals[7].

In spite of the presence of several commercially available serological tests for the diagnosis of acute toxoplasmosis, they have several disadvantages. As the parasite-specific antibodies may not be present during the early infection, false negative results particularly in immunosuppressed patients and in pregnant females can occur[8,9]. Moreover, false-positive results in which IgM antibodies persist for a long period post infection may represent a great dilemma in congenital toxoplasmosis[10]. Therefore, an ideal method which does not depend on antibody detection is urgently needed[7].

Recently, it was found that circulating microRNAs (miRNAs) in the plasma or serum can be used in the diagnosis of cancers. Additionally, it was found that such circulating miRNAs could serve as biomarkers for the detection of infectious diseases as schistosomiasis and malaria[11,12].

miRNAs are 18–22 nucleotide bases, endogenous regulatory factors that function to modulate cell differentiation and development[3]. They have the ability to regulate gene expression at the post-transcriptional level and are now considered as a key mechanism of gene regulation[13]. It was found that miRNAs released from a parasite are essential for cellular invasion, development and ability to respond to environmental and developmental signals. Moreover, they play an important role in modification of the host cellular environment for the parasite's own benefit, fitness and immune evasion[13–15]. In 2013, Hirugnanam *et al.*, concluded that toxoplasmosis may result in the initiation and progression of brain cancers through modifying the miRNA expression of human brain cells[16]. Several studies showed the significance of using plasma miRNA as biochemical markers for early detection of different types of human cancers, as colorectal, lung, pancreatic and prostatic cancers[17].

In contrary to usual RNA, miRNAs are stable enough to be detected in the plasma sample using quantitative time PCR (qRT-PCR)[18].

Researchers who tried to identify the *T. gondii* specific miRNAs found that miRNA712_3p is specifically expressed from the host cell during the process of host cell invasion by the parasite[7,19].

In this study, real-time PCR was used to measure the level of miRNA712_3p in the plasma of immune-competent and immune-compromised mice acutely infected with 2 500 tachyzoites of the virulent RH *T. gondii* strain, at different post-infection durations.

2. Materials and methods

2.1. Mice grouping and infection

Virulent RH HXGPRT (-) strain of *T. gondii* used in this study is routinely maintained in the laboratory of Medical Parasitology Department, Faculty of Medicine, Alexandria University, Egypt, which is cultivated by serial intraperitoneal passages of *T. gondii*

tachyzoites into mice. A total of 36 male Swiss strain albino mice were divided equally into three main groups: I (normal, non-infected control group), II (immune-competent group) and III (immune-compromised group). Immune-suppression was induced by intraperitoneal injection of the anticancer drug, endoxan, in two doses (70 mg/kg each) one week apart[20]. Two days after the last dose of endoxan, mice were injected intraperitoneally with 2 500 tachyzoites of the virulent RH strain. Three and five days post infection mice from different groups were sacrificed by euthanasia using terminal anesthesia maneuver. The experimental design followed the guidelines of laboratory animal care, according to the Ethical Committee, Faculty of Medicine, Alexandria University, based on international regulations of animal care (IRB NO: 00007555-FWA NO: 00018699). Infection was confirmed by Giemsa staining of peritoneal fluid after mice sacrifice[8].

2.2. Plasma sample collection

Immune-competent and immune-compromised groups were subdivided into two equal subgroups. Two subgroups were sacrificed three days post infection (II a & III a), while the other subgroups were sacrificed five days post infection (II b & III b). Sacrificed mice were exsanguinated, and blood was mixed with heparin for plasma separation. Blood samples were centrifuged at 1 200 g/min for 10 min, at room temperature, to remove the blood cells, followed by a second centrifugation at 12 000 g/min for 10 min, at 4 °C to remove other cellular components. Plasma samples were then stored at 80 °C until further processing[7].

2.3. Parasite count

T. gondii tachyzoites were counted in impression smears from livers of the sacrificed mice of all subgroups, at different post-infection durations[8].

2.4. RNA isolation

Total plasma RNA was isolated using miRNeasy Mini Kit (QIAGEN), according to the manufacturer's protocol. Briefly, synthetic cel-miR-39 was added to each sample as a spike-in control, then total RNA was purified from 400 µL of plasma sample. The miRNeasy Mini Kit contains phenol/guanidine-based lysis of samples and silica membrane-based purification of total RNA. Samples were homogenized in QIAzol lysis reagent. After addition of chloroform, the homogenate was separated into aqueous and organic phases by centrifugation. RNA partitions to the upper aqueous phase, while DNA partitions to the interphase and proteins to the lower organic phase or the interphase. The upper aqueous phase was extracted, and ethanol was added to provide appropriate binding conditions for all RNA molecules from 18 nucleotides (nt) upwards. The sample was then applied to the RNeasy mini spin column, where the total RNA binds to the membrane and phenol

and other contaminants are efficiently washed away. High quality RNA is then eluted in RNase-free water. The concentration of total RNA samples from plasma was quantified by a Nanodrop 2000 (Nanodrop, USA). The range of the result was from 11.9 to 73.7 ng/ μ L[7].

2.5. Real-time quantitative reverse-transcription PCR (RT-PCR)

The TaqMan® MicroRNA Reverse Transcription Kit supplied by Applied Biosystem was used for the reverse transcription reaction. The recommended reaction volume was 20 μ L. The plate was prepared so ABI prism 7900 sequence detection system (Ambion, USA) could be used for amplification and detection. Differences in miRNA712_3p were normalized to cel-miR-39, determined with the Δ Ct method, and reported as $2^{-\Delta\Delta C_t}$ [7].

2.6. Statistical analysis

Quantitative data of the present work was analyzed, using *F*-test (ANOVA) and *post hoc* test (Scheffe) for pairwise comparison. $P < 0.05$ was considered statistically significant. All statistical calculations were performed using IBM SPSS software package version 20.0.

3. Results

The aim of the present study was to investigate whether infection by *T. gondii* can drive changes to murine miRNA plasma levels in both immune-competent and immune-compromised mice several days post infection, or not.

To test our hypothesis, miRNA712_3p level in plasma samples from infected mice were profiled, three and five days post infection in the four studied subgroups in comparison to the non-infected control group.

3.1. miRNA712_3p is up-regulated in mice infected with *T. gondii*

RT-PCR results revealed that miR-712_3p level was significantly up-regulated in plasma samples of infected immune-competent subgroups (II a & II b), at 26.1 & 54.0 folds, three and five days post infection respectively (Table 1). A statistically significant difference was evident between the infected mice and the non-infected control group.

At the same time, the immune-compromised subgroups (III a & III b) showed up-regulation of the specific miRNA level to 4.0 and 8.8 folds, three and five days post infection respectively. The difference between the immune-compromised subgroups was statistically significant (Table 1). Moreover, the increase in the miRNA level in all studied subgroups was revealed to be statistically significant compared to the control non-infected mice.

Table 1

Comparison between different studied subgroups according to level of miRNA712_3p levels in mice plasma sample.

Group	MiR-712-3P level (range)	P1	P2	P3
I	0.014 2 \pm 0.013 2 (0.001 3-0.032 0)			
II a	0.370 4 \pm 0.252 0 (0.127 0-0.669 0)	0.001		
II b	0.766 3 \pm 0.105 6 (0.530 0-0.840 0)	0.001	0.003	
III a	0.056 5 \pm 0.023 2 (0.001 2-0.071 6)	0.031		0.001
III b	0.125 1 \pm 0.171 3 (0.015 0-0.397 8)	0.008	0.011	0.001

Data were expressed as Mean \pm SD. P1: comparison between group I and other subgroups, P2: comparison between group II a and group II b (group III a and group III b), P3: comparison between group II a and group III a (group II b and III b).

3.2. Parasite count in liver impression smears

The parasites were counted, three and five days post-infection. Tachyzoites of RH-infected subgroups were observed intracellular and extracellular. The mean tachyzoite count in impression smears obtained from immune-competent subgroups (II a) in the third-day post-infection was (0.783 \pm 0.343) tachyzoites/oil immersion field (OIF). The count showed 4.7 folds increase by the fifth-day post infection (3.667 \pm 2.059) tachyzoites/OIF, and the difference in parasite count was found to be statistically significant (Table 2).

As regards the immune-compromised subgroups (III a & III b), the mean tachyzoite count was (0.533 \pm 0.308) tachyzoites/OIF, three days post infection. Five days post-infection, the mean tachyzoite count showed an increase up to 1.9 folds. The difference in the mean count was found to be statistically significant, three and five days post infection (Table 2).

Table 2

Comparison between different studied infected subgroups regarding *Toxoplasma* tachyzoites count in liver impression smears three and five days post infection.

Group	Tachyzoites count (range)	P1	P2
II a	0.783 \pm 0.343 (0.500-1.400)		
II b	3.667 \pm 2.059 (1.200-9.500)	0.001	
III a	0.533 \pm 0.308 (0.200-1.000)		0.108
III b	1.000 \pm 0.424 (0.600-1.800)	0.013	0.003

Data were expressed as Mean \pm SD. P1: comparison between II a and II b (III a and III b), P2: comparison between II a and III a (II b and III b).

4. Discussion

miRNAs are small RNA sequences that can regulate gene expression at the post-transcriptional level[21]. They can exert their action by targeting protein-encoding messenger RNAs (mRNA)[22]. Several studies reported their role in diverse biological aspects within the cell, including organism development, immune modulation, metabolism and cell death[23-25].

In recent years, it was evident that plasma miRNA can be used as

a promising tool for diagnosis of several diseases[26-28]. In 2008, miRNA was used for the first time in tumor diagnosis[17]. In the same year, Lawrie *et al.* reported tumor associated miRNA as a non-invasive diagnostic tool of diffuse B-cell lymphoma[29]. Thereafter, it was used in diagnostic trials for other cancers[30].

In 2012, Wang *et al.* found an evidence for the differential expression of miRNAs in the two genetically distinct strains of *T. gondii*, RH virulent and Me 49 avirulent strain[3]. Among over 1 000 miRNAs investigated, miR-132 was the only one that was up-regulated more than 2 folds increase, by the three different *T. gondii* strains[31,32]. In 2014, two different mature forms of *T. gondii* specific miR-712 were recorded, 712-5p and miR-712-3p. These miRNAs were reported to be specific for RH strain, but their role as a diagnostic marker for toxoplasmosis in immunosuppression was still unknown[7].

This study was carried out on plasma samples collected from mice previously infected with 2 500 tachyzoites of RH strain. The samples were processed and preserved under -80 °C till RNA extraction was carried out according to Aiso *et al.*, who reported that freezing of the plasma sample recorded a better detection of miRNAs[33].

In the present work, detection of circulating miRNA712_3p in the plasma of *T. gondii*-infected mice was feasible. miRNA712_3p level significantly has been up-regulated in all infected subgroups (II a, II b, III a & III b) in comparison to non-infected control group (I), with 54.0 folds increase in the immune-competent mice, five days post infection. This may be due to the ability of *T. gondii* to control host cell microenvironment. This control measure occurs through channelizing parasite proteins into the host cell cytoplasm through the parasitophorous vacuole. Some of the proteins are targeted to the host nucleus regulating gene expression at the post-transcriptional level, leading to transcription of regulatory microRNAs specific to these parasites[34].

Both miRNA 712_3p and *T. gondii* parasite activity are greatly influenced by the process of Ca²⁺ mobilization within the cell[35]. *T. gondii* depends on Ca²⁺ metabolism in both movement and invasion of the host cell[35]. This may be the main mechanism which can explain the high increase in the level of miRNA 712_3p in infected mice in direct association with the duration of infection, due to the increase in the number of moving, replicating and invasive parasites[35].

In agreement with our results, Jia *et al.* reported an up to 80.62 folds increase in the level of several miRNAs in plasma of mice, 72 h after intra peritoneal infection with 106 tachyzoites of RH strain compared to control non-infected mice[7]. The higher increase in the latter study could be attributed to the variation in the infective dose.

The fold increase in the plasma miRNAs level was significantly higher in immune-competent subgroup than in the immune-compromised one on the third and fifth days post infection respectively. This difference may be due to the impact of regulatory miRNA on the immune response, as they act as mediators for both innate and acquired immune responses[36]. In 2011, Tserel *et al.* reported the role of miRNAs on control of macrophage production and activation, noticing that macrophages are the main host cell needed by the parasite, and determining the multiplication power of the parasite within it. The authors reported the role of microRNAs in the regulation of eosinophilic recruitment in case of *T. gondii* induced eosinophilic meningitis[37]. Down-regulation of the main

host cell recruitment reduces the multiplication power of the parasite leading to a reduction in the secreted proteins which in-turn stimulate the production of miRNAs, and so on. Talari *et al.*, in 2017, reported that ectopic expression of miR-712 impaired the secretion of pro-inflammatory cytokines such as TNF- α , IL-6, and IFN- β , thus, decreasing recruitment of inflammatory cells and macrophages[38].

It is worth mentioning that, the parasite and the host cell have the ability to produce several regulatory miRNAs, and the end result of their battle will determine which one can predominate over the other[39].

The mean tachyzoite count in Giemsa stained liver impression smears was considered as an indicator for progression of infection. Therefore, in the present study, the tachyzoite load in the liver was studied to demonstrate the role of parasite multiplication on the variations in micRNA 712_3p level.

There was a statistically significant increase in the mean tachyzoite count in the studied infected subgroups sacrificed on the fifth day (3.667 \pm 2.059) tachyzoites/OIF, in comparison to the other subgroup, sacrificed on the third-day post infection (0.783 \pm 0.343) tachyzoites/OIF. This result was in agreement with Mahmoud[40] who also used Giemsa stain impression smear as a parasitological parameter for drug efficacy; the mean tachyzoite count in the control subgroup infected with 2 500 tachyzoites was (6.27 \pm 2.67) tachyzoites/OIF on the seventh-day post infection denoting an increase in the parasite load with time[40]. In the present work, the increase in the mean tachyzoites count was found to be more evident in the immune-competent subgroup sacrificed on the fifth day in comparison to the immune-compromised subgroup sacrificed on the same day. The difference in parasite count between immune-competent and immune-compromised subgroups may be due to reduction in number of the main host cell, macrophages, induced by endoxan, hence, decreasing the multiplication power of the parasite. Therefore, it was noted that the increase in the level of miRNA was in association with the increase in the parasite density, as detected by liver impression smears.

The present work noted the significant up-regulation of *T. gondii* specific microRNA 713-3p in murine plasma samples, few days post infection, in both immune-competent and immune-compromised mice. Thus, it raises hope for opening a new scope for early diagnosis of acute toxoplasmosis.

Conflict of interest statement

We declare that we have no conflict of interests.

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