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Original Article

Expression of Mir-21 and Mir-103a in *Toxocara canis*: Potential for Diagnosis of Human Toxocariasis

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Abstract

Background: Toxocariasis is one of the most neglected zoonotic diseases, predominantly caused by *Toxocara canis*. We aimed to evaluate the expression of microRNAs 21 and 103a in seropositive individuals for human toxocariasis as diagnostic biomarkers.

Methods: This study was conducted on 324 individuals for ELISA test on toxocariasis in Tehran and Karaj, Iran 2019. Then positive samples for anti-*Toxocara* IgG were obtained to quantitative Real-time PCR (qRT-PCR) assays to investigate the transcriptional profiles of miRNAs predicted to be involved in developmental and reproductive processes. qPCR was employed to assess levels of transcription for miRNAs of 103a and 21 in plasma samples.

Results: After the experiments, the results were evaluated by REST software, Livak formula and quantitative *t*-test. The analyzes performed on human samples showed that in the case group compared to the control group, only in *Tc*-miR-21 gene, a 0.3-fold increase in expression was obtained with REST software (Fold change ≤ 1.5, $P>0.05$), which was statistically significant by *t*-test ($P<0.05$).

Conclusion: To our knowledge, this is the first study to evaluate miR-21 and miR-103a in toxocariasis, which shed light on the fundamental role of it as a biomarker and diagnostic tool. However, due to the changes in expression of these miRNAs were not vast to be used as biomarkers in diagnosis. Despite of that the changes in the expression of these miRNAs were not vast but they could serve as novel promising biomarkers for diagnosis of toxocariasis.

Introduction

Toxocara species are one of the most prevalent zoonotic helminths, and are an intestinal nematode of dogs (*T. canis*) and cats (*T. cati*), which are the causative agent of human infection globally (1, 2). Humans, particularly children, may be infected by accidental ingestion of embryonated *Toxocara* eggs from contaminated soil, water, raw vegetables, fruit or infective larvae in raw/undercooked meats (3). After ingestion of embryonated eggs, larvae can invade multiple tissues or organs, causing visceral larvae migrans (VML), ocular larva migrans larvae (OLM), neurotoxocariasis or covert toxocariasis (1, 4). Globally, the prevalence of *Toxocara* spp. is varying and about one fifth (19%) of the world's human population is seropositive to *Toxocara*. Africa showed the highest seroprevalence rates (37.7%) and the lowest in the Eastern Mediterranean region (8.2%) (5). Misdiagnosis of toxocariasis because of nonspecific clinical presentation, may lead to prolonged morbidity and health complications. The diagnosis based on clinical, radiographic and laboratory investigations of the disease (6, 7). *Toxocara* diagnostic methods in humans include histopathological examination, or detection of specific larval DNA and the main diagnostic method is the serological or immunological for detection of anti-*Toxocara* antibodies (IgG) (3).

Recently, molecular methods and the knowledge of the genome and transcriptomes play a fundamental role in understanding the molecular biology, biochemistry and physiology of *Toxocara* (8, 9). MicroRNAs (miRNAs) were first detected in a free-living nematode; these small RNAs are post-transcriptional regulators of gene expression and showed association with different immune-related diseases (10). The quantitative detection of miRNAs by polymerase chain reaction (PCR) in different body fluids such as blood, urine and saliva has increased the ability for using as biomarkers and diagnostic tools (11). The identification of miRNAs in biological samples, easily collected and their stability improve their use as a diagnostic tool for infectious parasitic diseases (11). Evaluating changes in the expression of different genes in different evolutionary stages of parasites can indicate their role in diagnosis, host-angel communication, and drug resistance. Therefore, for some reason, including an increase in the number of miRNAs detected in parasites, the presence of circulating miRNAs derived from worms in helminthic infection, and their persistence in host samples, the change in miRNAs expression in host or parasite should be considered more serious for diagnostic and therapeutic purposes (12).

The objectives of the present study were to evaluate the expression of *T. canis* miR-21 (*Tc*-miR-21) and *T. canis* miR-103a (*Tc*-103) and using it as a biomarker and diagnostic tool for human toxocariasis in the people with anti- *T. canis* immunoglobulin G.

Materials and Methods

Ethics statement

All tests on individuals were carried out according to the guidelines of the Tehran University of Medical Sciences Ethics Committee (Ethics ID # IR.TUMS.SPH.REC.1398.096).

Subjects

This study was conducted on 324 individuals referred to the Teaching Hospitals of Alborz University of Medical Sciences, Karaj, Iran in 2019. Whole blood samples were centrifuged (1500 g for 10 min). The isolated plasma was again centrifuged (1500 g for 5 min). Finally, plasma samples were stored at -80 ºC until analyses were carried out. Plasma samples had the ability to isolate and detect more specific miRNAs than serum and whole blood sam-

ples due to the lack of stress of coagulation reactions (13).

Detection of anti-Toxocara canis immunoglobulin G *by indirect ELISA*

Anti-*T. canis* antibodies were detected by commercial ELISA kit (NOVATEC GmbH, Dietzenbach, Germany). The specificity and sensitivity of the kit was more than 95%, following the manufacture's instruction. In brief, the wells of 96 well microtiter plates were coated with *Toxocara canis* antigens in coating buffer (0.06 M carbonate buffer, pH 7.2). Plasma samples (1:100 diluted in PBS; 100 µl/well) were dispensed in duplicates and incubated (at 37 ºC for 1 hour). After this step, each well was washed 3 times (PBS; 1:20 diluted 0.2 M pH=7.2). In next step, wells were incubated with HRP conjugated anti-IgG antibodies (100 µl/well) for 30 min at 25 ºC. Finally, wells were washed five times by addition of TMB/H_2O_2 substrate (100 µl/well) the color was developed. After 15 min of incubation, reaction was stopped by addition of H_2SO_4 (2 N; 50 µl/well) and the optical density was measured at 450 nm. All plasma samples were stored at -80 ºC for molecular analysis.

Quantitative Real-time PCR (qRT-PCR) assays

To investigate the transcriptional profiles of miRNAs predicted to be involved in developmental and reproductive processes, qRT-PCR was employed to assess levels of transcription for miRNAs *Tc*-miR-21 and *Tc*-miR-103a in 60 plasma samples (30 control samples and 30 case samples). Total RNAs was extracted separately from the plasma samples using TRIzol (TRI reagent). The extracted RNAs were approved by qualitative (agarose gel electrophoresis) and quantitative (NanoDrop spectrophotometer) methods. Then, RNAs were polyadenylated and transcribed into first-strand cDNA, according to the manufacturer's protocol in kit (BON-Stem miR cDNA synthesis, Iran). To estimate transcription levels, a two-step qPCR was carried out using a transcript BON-miR qPCR Kit employing the following cycling protocol: 95 °C hold for 2 min, 95 °C for 5 sec, followed by 40 cycles of 60 °C for 30 sec. The oligonucleotide probes used for labeling and hybridization are as shown in Table 1.

Table 1: Sequences of primers used for qRT- PCR amplification

Primers	*Sequences*	*References*
Tc-miR-21 (Forward)	ACGTGTTAGCTTATCAGACTGA	[36]
Tc-miR-21 (Stem loop RT primer)	GTCGTATGCAGAGCAGGGTCCGAGGTATTCGCACTGCATACGACTCAAC	[34]
Tc-miR-103a-3P (Forward)	UACAGUACUGUGAUAACUGAA	[34]
Tc-miR-103a-3P (Stem loop RT primer)	GTCGTATGCAGAGCAGGGTCCGAGGTATTCGCACTGCATACGACTTCAGT	[34]
U6 (Forward)	GCTTCGGCAGCACATATACT	[34]
Reverse (Universal)	GAGCAGGGTCCGAGGT	[36]

Based on studies conducted, *U6* housekeeping gene (HKG) was selected in this study for normalization (14-16).

Statistical analysis

Two independent replicates were performed, and the relative transcription level was established using the Livak formula ($2^{-\Delta\Delta Ct}$; CT: threshold cycle), relative expression software tool (REST 2009) and *t*-test (17-21). Statistical evaluations were performed with SPSS (ver. 19.0, Chicago, IL, USA). Chi-square test and Fisher's exact test were used for categorical data. A *P*-value<0.05 was considered statistically significant.

Results

ELISA

Of 324 plasma samples, 30 were positive (anti-*T. canis* IgG) by ELISA technique (9.2%). The average age of the positive cases was 45.3 and there was no significant difference between gender and infection (17 males and 13 females, *P*=0.997).

Real-time PCR

The expression of *Tc*-miR-21 by Real-time PCR method in patients with anti-*T. canis* IgG (cases) and the people without anti-*T. canis* antibodies (controls) plasma samples with ELISA shown in Table 2 and Fig. 1. Moreover, the case and control samples matched in terms of age and gender.

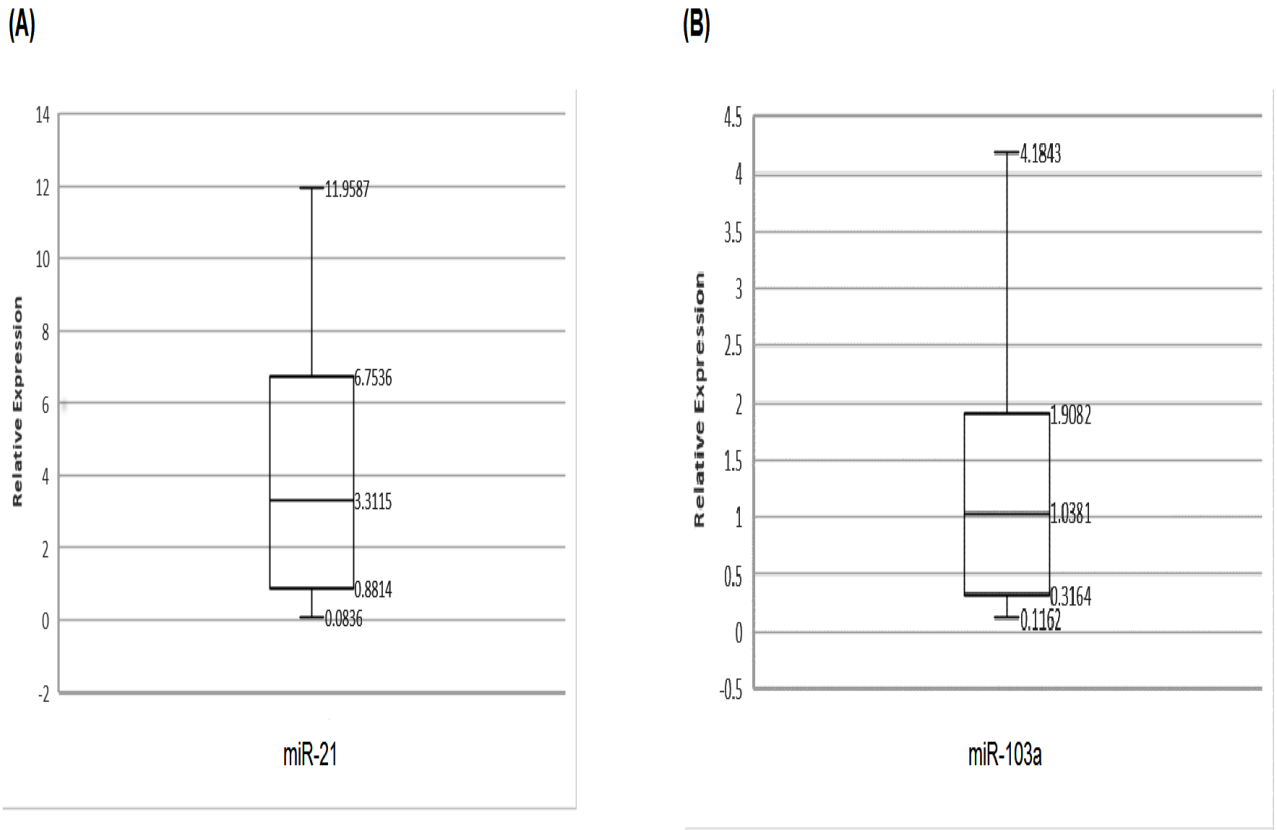

Fig. 1: The expression of miRNAs using plasma samples and qRT-PCR in the people with (cases) and without (controls) anti-*Toxocara canis* immunoglobulin G based on REST. A) *Tc*-miR-21, and B) *Tc*-miR-103a

Table 2: The expression of Tc-miR-21 in the people studied using plasma samples and Real-time PCR based on Livak formula

CASE	*Tc-miR-21 (avg)*	*U6 (avg)*	*Δ Ctcase Tc-miR-21-U6*	*CONTROL*	*Tc-miR-21 (avg)*	*U6 (avg)*	*ΔCtcontrol Tc-miR-21-U6*	*ΔΔCt*	*Fold change*
H1a	33.23	28.05	5.19	C1a	34.90	26.89	8.02	-2.83	7.110741
H2a	33.23	26.45	6.78	C2a	36.00	27.21	8.79	-2.01	4.027822
H3a	33.88	26.80	7.09	C3a	34.90	27.06	7.84	-0.76	1.687632
H4a	32.59	26.44	6.15	C4a	34.95	26.58	8.38	-2.23	4.675109
H5a	32.21	27.06	5.15	C5a	35.35	27.27	8.09	-2.94	7.647563
H6a	32.27	26.69	5.59	C6a	35.01	26.18	8.83	-3.25	9.480742
H7a	32.04	24.93	7.12	C7a	35.56	26.80	8.76	-1.64	3.116658
H8a	33.04	25.19	7.85	C8a	33.85	25.46	8.39	-0.54	1.448942
H9a	34.51	25.93	8.58	C9a	35.13	29.60	5.53	3.05	0.121161
H10a	34.50	25.88	8.63	C10a	34.10	26.02	8.09	0.54	0.687771
H11a	33.77	25.62	8.15	C11a	34.23	26.88	7.35	0.81	0.572362
H12a	33.90	25.66	8.24	C12a	34.23	23.26	10.97	-2.73	6.634556
H13a	34.34	26.20	8.14	C13a	34.90	23.91	10.10	-2.86	7.235035
H14a	31.00	26.18	4.83	C14a	35.00	27.66	7.34	-2.52	5.715977
H15a	35.11	26.38	8.73	C15a	34.06	26.87	7.19	1.54	0.343885
H16a	34.95	26.75	8.20	C16a	34.75	23.67	11.08	-2.88	7.336032
H17a	36.10	26.80	9.30	C17a	35.90	26.68	9.22	0.08	0.946058
H18a	32.88	26.49	6.39	C18a	36.53	26.56	9.97	-3.58	11.95879
H19a	33.03	27.00	6.03	C19a	34.15	25.57	8.59	-2.56	5.876675
H20a	32.01	26.87	5.14	C20a	34.55	25.15	9.40	-4.26	19.09337
H21a	34.65	26.77	7.88	C21a	34.99	25.90	9.09	-1.21	2.305373
H22a	33.80	26.05	7.75	C22a	34.17	26.31	7.87	-0.12	1.086735
H23a	32.33	23.99	8.34	C23a	33.68	25.25	8.43	-0.09	1.06437
H24a	33.47	26.49	6.98	C24a	35.00	26.21	8.79	-1.81	3.506423
H25a	35.76	26.92	8.84	C25a	37.06	25.88	11.18	-2.34	5.04551
H26a	34.92	23.25	11.68	C26a	35.24	26.00	9.24	2.44	0.184923
H27a	33.53	24.11	9.42	C27a	34.17	26.46	7.71	1.71	0.30566
H28a	32.20	25.68	6.52	C28a	35.52	27.00	8.52	-2.00	3.986161
H29a	36.04	26.56	9.48	C29a	34.74	24.13	10.61	-1.13	2.181015
H30a	35.83	24.35	11.48	C30a	34.27	26.37	7.90	3.58	0.08362

Tc-miR-21 expression is UP -regulated in the case group (in comparison to control group) by a mean factor of 3.311. *Tc*-miR-21 case group was different to control group [P (H1): 0.035; SE: 0.332-24.556; 95% CI: 0.035-11.279]. Thus, the division of housekeeping expression (*U6*) into gene expression (*Tc*-miR-21) indicated the rate of change (⁄3.311=+0.302). In addition, analyzed results by *t*-test showed that there was a significant relationship between gene expression in the two groups (t-Critical two-tail: 2.001 < t-Stat: 4.198; Pooled Variance: 1.099; $P<0.05$).

Table 3 and Fig. 1 shows the expression of *Tc*-miR-103a in sero-positive and sero-negative samples by Real-time PCR. Moreover, the case and control samples matched in terms of age and gender. The present findings showed that *Tc*-miR-103a expression was the same in both groups [Expression: 1.038; SD: 0.278-2.480; 95% CI: 0.110-8.240; P (H1): 0.505]. Thus, the division of HKG expression (*U6*) into gene expression (*Tc*-miR-103a) indicated the rate of change (⁄1.038~1). In addition, analyzed results by t-test showed that there was not a significant relationship between gene expression in the two groups (t-Critical two-tail: 2.001 > t-Stat: 0.056; Pooled Variance: 1.792; $P > 0.05$). Moreover, changes in the expression of *Tc*-miR-21 and *Tc*-miR-

103a in all case samples, based on the analysis of previous studies, were not significantly correlated (Expression ≤ 1.5, Fold change ≤ 5, $P \geq 0.05$).

Table 3: The expression of Tc-miR-103a in the individuals studied using plasma samples and Real-time PCR based on Livak method

CASE	*Tc-miR-103a (avg)*	*U6 (avg)*	*ΔCtcase Tc-miR-103a-U6*	*CONTROL*	*Tc-miR-103a (avg)*	*U6 (avg)*	*ΔCtcontrol Tc-miR-103a-U6*	*ΔΔCt*	*Fold change*
H1a	35.6	28.05	7.56	C1a	34.90	26.89	8.02	-0.46	1.375542
H2a	35.12	26.45	8.67	C2a	36.00	27.21	8.79	-0.12	1.086735
H3a	34.73	26.80	7.94	C3a	34.90	27.06	7.84	0.10	0.936272
H4a	34.56	26.44	8.12	C4a	34.95	26.58	8.38	-0.26	1.193336
H5a	35.16	27.06	8.11	C5a	35.35	27.27	8.09	0.02	0.986233
H6a	34.62	26.69	7.93	C6a	35.01	26.18	8.83	-0.90	1.866066
H7a	33.25	24.93	8.33	C7a	35.56	26.80	8.76	-0.43	1.347234
H8a	34.33	25.19	9.14	C8a	33.85	25.46	8.39	0.76	0.592546
H9a	34.57	25.93	8.64	C9a	35.13	29.60	5.53	3.11	0.116226
H10a	35.00	25.88	9.13	C10a	34.10	26.02	8.09	1.04	0.486327
H11a	36.00	25.62	10.38	C11a	34.23	26.88	7.35	3.04	0.122004
H12a	35.60	25.66	9.94	C12a	34.23	23.26	10.97	-1.03	2.042024
H13a	34.69	26.20	8.49	C13a	34.90	23.91	11.00	-2.51	5.676493
H14a	35.36	26.18	9.19	C14a	35.00	27.66	7.34	1.85	0.278355
H15a	36.24	26.38	9.86	C15a	34.06	26.87	7.19	2.67	0.157127
H16a	37.00	26.75	10.26	C16a	34.75	23.67	11.08	-0.82	1.765406
H17a	35.30	26.80	8.50	C17a	35.90	26.68	9.22	-0.72	1.647182
H18a	35.26	26.49	8.78	C18a	36.53	26.56	9.97	-1.20	2.289448
H19a	34.56	27.00	7.56	C19a	34.15	25.57	8.59	-1.03	2.034959
H20a	34.33	26.87	7.46	C20a	34.55	25.15	9.40	-1.94	3.823781
H21a	35.87	26.77	9.10	C21a	34.99	25.90	9.09	0.02	0.989657
H22a	34.49	26.05	8.44	C22a	34.17	26.36	7.87	0.58	0.671286
H23a	33.25	23.99	9.26	C23a	33.68	25.25	8.43	0.83	0.562529
H24a	36.90	26.49	10.42	C24a	35.00	26.21	8.79	1.63	0.32421
H25a	35.32	26.92	8.40	C25a	37.06	25.88	11.18	-2.78	6.84476
H26a	34.26	23.25	11.01	C26a	35.24	26.00	9.24	1.77	0.293209
H27a	34.11	24.11	10	C27a	34.17	26.46	7.71	2.29	0.204476
H28a	33.90	25.68	8.22	C28a	35.52	27.00	8.52	-0.30	1.231144
H29a	35.10	26.56	8.54	C29a	34.74	24.13	10.61	-2.07	4.18434
H30a	35.03	24.35	10.69	C30a	34.27	26.37	7.90	2.79	0.145088

Discussion

In the past several years, an impressive research effort has been directed toward identification and role of miRNAs in parasites (including nematodes). Circulating miRNAs in body fluids have been considered as potentially valuable biomarkers in diagnosing, treatment and follow-up in diseases (22). MiRNAs, which are relatively stable in the circulatory system, may serve as molecular valuable biomarkers for identification of disease status (23). In recent years, intracellular miRNAs or tissues are found to be extracellular and biologically active, such as plasma, urine, CNS and saliva (24).

The use of miRNAs in the diagnosis of parasitic helminths infections has been proven (25-28). However, genomic, transcriptomic and proteomic investigations on parasitic nematodes such as *Toxocara* are in early stages due to the lack of recognition of the role of miRNAs in parasites and hosts. Several studies

worldwide have examined the miRNAs in parasites with assigning a specific role to them. MiRNAs may have a role in drug resistance in nematodes (29). Diversity was studied in parasitic nematode genomes (30). MiRNAs' expression might reflect adaptations to different lifestyles and environments. A previous study showed the expression of miR-36 of filarial nematode and its effect on the life cycle of *Brugia malayi* (31). MiRNAs can have a role of adaptation to the life cycle and pathogenicity of *Ascaris* (32). In the following, studies conducted on *T. canis* revealed that there are 342 conserved miRNAs. The miRNAs of miR-2305 and miR-6090 play a regulatory role in reproduction, embryo and larval development, let-7-5P, miR-34, and miR-100 appeared to be involved in host-parasite interactions. The common markers such as miR-2861, miR-2881, and miR-5126 in *T.* might have implications for the prediction of drug resistance (33, 34). The expression of miR-21 and miR-103a in the larval stage of *T. canis* have been increased and decreased in comparison with eggs and adults' stages of the parasite, respectively.

These miRNAs may have a role in the maturation and development of *T. canis* (Unpublished data). In addition, these miRNAs were identified using reputable sites such as miRNAs and NBC (35). Therefore, we decided to investigate the potentials of *Tc*-miR-21 and *Tc*-miR-103a for the diagnosis of human toxocariasis.

Conclusion

The present study is the first, to the best of our knowledge, to analyze the circulating miR-21 and miR-103a in human toxocariasis. Although increased expression of miR-21 was observed in case study samples, due to gene expression changes less than 1.5-fold in case samples, miR-21 and miR-103a in the diagnosis of human toxocariasis, cannot have the full characteristics for introducing as a diagnostic biomarker.

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Conflict of interest

The authors declare that there is no conflict of interest.

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