Research paper

Circulating miRNAs as footprints for liver fibrosis grading in schistosomiasis

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Abstract

Background: Chronic infection with Schistosoma japonicum or S. mansoni results in hepatic fibrosis of the human host. Staging fibrosis is crucial for the prognosis and to determine the rapid need of treatment in patients with schistosomiasis.

Methods: To establish whether there is a correlation between circulating microRNA (miRNA) level and fibrosis progression in schistosomiasis, ten miRNAs were selected to assess their potential in grading schistosomiasis liver fibrosis. This was done firstly in two mouse strains (C57BL/6 and BALB/c) to determine the temporal expression profiles in serum over the course of S. japonicum infection, and then within a cohort of 163 schistosomiasis japonica patients with different grades of liver fibrosis.

Finding: Four miRNAs (miR-150-5p, let-7a-5p, let-7d-5p and miR-146a-5p) were able to distinguish patients with mild versus severe fibrosis. The level of serum miR-150-5p showed the most promising potential for grading hepatic fibrosis in schistosomiasis. The diagnostic performance of miR-150-5p in discriminating mild from severe fibrosis is comparable with that of the ELF test and serum HA level. In addition, the serum levels of the four miRNAs rebounded in infected C57BL/6 mice, after 6 months post treatment, following the regression of liver fibrosis, thereby providing further support for the utility of these miRNAs in grading schistosomal hepatic fibrosis.

Interpretation: Circulating miRNAs can be a supplementary tool for assessing hepatic fibrosis in human schistosomiasis.

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1. Introduction

Schistosomiasis, a major neglected tropical disease of poverty, affects >230 million people worldwide [11]. Three species of schistosomes (trematode blood flukes), Schistosoma mansoni, S. haematobium and S. japonicum, are the most clinically relevant. The annual number of disability-adjusted life years (DALYs) lost for this disease has been estimated to be up to 3.5 million in 2015 [33]. Currently, the control of the disease predominantly relies on mass drug administration (MDA) programs with praziquantel (PZQ).

Chronic infection with S. japonicum or S. mansoni, known as hepatosplenic schistosomiasis, exhibits clinical pathology features comprising granuloma formation, periportal fibrosis, portal hypertension, hepatosplenomegaly, ascites, and the formation of vascular shunts [7]. The hepatic pathology during hepatosplenic schistosomiasis is triggered by soluble egg antigen (SEA) secreted from mature eggs lodged in host liver tissue. Immune cells recruited to the periphery of eggs create a type 2 cytokine (interleukin 4 (IL-4) and IL-13)-enriched niche, which leads to the granuloma formation, macrophage differentiation, hepatic stellate cell (HSC) activation and then fibrosis development. The liver residential cells, HSCs, and recruited immune cells (eosinophils, neutrophils, macrophages, and lymphocytes) have been identified as major contributors to these cellular events [10].

MicroRNAs (miRNAs) are small non-coding RNAs (18–25 nt), which act as subtle gene expression regulators of a variety of cellular processes, including fibrogenesis [19,38]. Several studies have shown the dysregulation of miRNAs in liver tissue during schistosome infection in both...
2. Materials and methods

2.1. Ethics statement

All work on animal was performed according to the Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition) and with the approval of the QIMR Berghofer Medical Research Institute Animal Ethics Committee (Ethics Approval: Project P288). Human study was approved by the Human Ethics Committee, QIMR Berghofer Medical Research Institute (QIMRB), Brisbane, Australia (Ethics Approval: Project PS24) and the Institutional Review Board of the Research Institute for Tropical Medicine, Department of Health, Manila, the Philippines (Institutional Review Board Number 2012-13-0), in accordance with the Declaration of Helsinki. Written informed consent was received from each study participant or from the legal guardians of those aged <15 years.

2.2. Study cohort

The human cohort study was carried out in 18 schistosomiasis-endemic barangays in the municipalities of Laonag and Palapag, Northern Samar, the Philippines. Detailed information of the study population is described elsewhere as part of a hepatic morbidity study [37,39]. We enrolled 163 individuals (all negative for HBV/HCV infection) from the cohort all of whom were assessed for liver fibrosis severity by ultrasound scan using a portable gray-scale ultrasonogram equipped with 3 MHz curve array transducer (SONAACE X1; Madison Co., Ltd., Seoul, South Korea). Hepatic fibrosis assessment was adopted from the practical standards established for ultrasonography by the WHO/TDR in 2000 (WHO Special Programme for Research and Training in Tropical Diseases). Ultrasound findings were determined by consensus agreement of two experienced ultrasonographers [37].

2.3. Mouse infection, serum collection, histological assessment and biochemical analyses

Eight-week-old female C57BL/6 and BALB/c mice were infected percutaneously with a low challenge dosage (16 ± 2) of S. japonicum cercariae. Mice were sacrificed at different time points post infection and ~1 ml blood was collected at each time point by cardiac puncture. The median lobe from each mouse liver was formalin-fixed and subjected to histological assessment [6]. Slides were scanned using the Aperio Slide Scanner (Aperio Technologies, Vista, USA). The percent of granuloma volume density (Gra) and of hepatic necrosis (Nec), and the percent of collagen staining (degree of hepatic fibrosis, HF) were quantified using ImageScope V10.2.1 (Aperio) with H&E and picrorous red stained slides, respectively. Liver hydroxyproline (HP) content was measured by a Hydroxyproline Colorimetric Assay Kit (Bios Scientific, Austin, TX). Serum alanine transaminase (ALT) and aspartate transaminase (AST) levels were determined using ALT and AST color endpoint assay kits (Bios Scientific, Austin, TX), respectively.

2.4. Mouse infection, drug treatment and sample collection

Three groups of eight-week-old female C57BL/6 mice (For each group, n = 8) were used in the experiment. One group of uninfected mice was used as control, while other two groups were percutaneously infected with 16 ± 2 S. japonicum cercariae. For the latter groups, one group mice were sacrificed at 6 weeks p.i., and blood and liver samples were collected. The other group of mice were orally administered 150, 200, 250, 300 and 350 mg/kg PZQ (Sigma, USA) prepared in 2.5% (v/v) Kolliphor EL (Sigma, USA) for 5 consecutive days [8], and blood and liver samples were collected, at 6 months post treatment (p.t.). Tubes containing blood were centrifuged at 3000g for 10 min at room temperature and the sera retained. Fecal egg burden was assessed at 6 weeks.
Fig. 1. Temporal abundance of serum miRNAs in two mouse strains during S. japonicum infection. (a) C57BL/6 mice, (week 0, n = 8; week 4, n = 7; week 6, n = 6; week 7, n = 6; week 9, n = 9; week 11, n = 7; week 13, n = 7). (b) BALB/c mice, (week 0, n = 10; week 4, n = 5; week 6, n = 7; week 7, n = 8; week 9, n = 9; week 11, n = 8). Boxes represent the interquartile range of the data. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. P values were calculated using One-way ANOVA. (ns = no significant difference).
Correlations were calculated by Pearson's correlation coefficient (r) for discriminating mild (grade 0-I) from severe (grade II-III) using the serum levels of miRNAs (or in combination) as potential markers for discriminating mild (grade 0-I) from severe (grade II-III) schistosomiasis hepatic fibrosis. In the PZQ treated schistosomiasis mouse model, the Mann-Whitney U test was used for analysis of fecal egg burden and liver collagen deposition between the two infected groups of C57BL/6 mice; a p-value of <0.05 was considered statistically significant. One-way ANOVA followed by Holm-Sidak multiple comparison was used for analysis of the serum levels of miRNAs between control, infected (6 weeks p.i.) and PZQ-treated mice (infected, treated at 6 weeks p.i. and tested at 6 months p.t.). Statistical analysis was performed with GraphPad Prism Version 6.00 for windows.

Further details about the study methods are provided in the Supplementary material.

### 3. Results

#### 3.1. Temporal expression of serum miRNAs in the two mouse strains during *S. japonicum* infection

Ten miRNAs were selected to test their potential for grading schistosomiasis liver fibrosis based on their correlation with virus-related hepatic fibrosis and/or myocardial fibrosis progression (Supplementary Table 1). Temporal expression of these ten miRNAs was initially investigated in two genetically different murine models of *S. japonicum* infection (Fig. 1a and b). In C57BL/6 mice, 4 of the 10 miRNAs (miR-200b-3p, let-7d-5p, miR-146a-5p and miR-222) were significantly down-regulated from 4 weeks p.i., except let-7d-5p at 7 weeks p.i. and onwards, while 2 of them (miRNA-150-5p and let-7a-5p) were significantly down-regulated from 6 weeks p.i. and onwards. In BALB/c mice, only miRNA-150-5p presented a similar expression pattern to that found in C57BL/6 mice across the infection course. The serum level of miR-200b-3p was significantly down-regulated at 6 weeks p.i. and onwards. The serum levels of miR-192a-5p and miR-185-5p was significantly increased at 6 weeks p.i. and onwards (except miR-185-5p at 11 weeks p.i.), while the levels of both miRNAs were not altered in C57BL/6 mice during the infection. Furthermore, the level of miR-29a-3p in BALB/c mice was significantly up-regulated at 4 weeks p.i. and onwards, while no statistically significant change in the serum level of miR-29a-3p was observed over the infection course in C57BL/6 mice. The global expression trend of these serum miRNAs in the two mouse strains during *S. japonicum* infection is presented as Supplementary Table 2.
3.2. Correlation of serum miRNA levels with pathological parameters during S. japonicum infection in mice

We then analyzed the correlations between the serum levels of the ten miRNAs and liver pathological parameters in both C57BL/6 and BALB/c mice (Table 1 and Fig. 2). The level of miR-192a-5p was most positively correlated with the levels of liver enzymes, followed by miR-29a-3p and miR-185-5p. In contrast, the level of miR-150-5p in serum was most inversely correlated with the levels of liver enzymes. The serum levels of miR-150-5p, miR-200b-3p and miR-146a-5p were inversely correlated with liver HP content, the intensities of hepatic fibrosis and granuloma in both mouse strains (in C57BL/6 mice, for miR-150-5p, $r = -0.5770$, $p < 0.0001$, $r = -0.5435$ and $p < 0.0001$, and $r = -0.5919$, and $P < 0.0001$; for miR-200b-3p, $r = -0.5249$, $p < 0.0001$, and $P < 0.0001$; for miR-146a-5p, $r = -0.5423$, $p < 0.0001$, and $P < 0.0001$; in BALB/c mice, for miR-150-5p, $r = -0.6530$, $p < 0.0001$, and $P < 0.0001$; for miR-200b-3p, $r = -0.6013$, $p < 0.0001$, and $P < 0.0001$; for miR-146a-5p, $r = -0.5529$, $p < 0.0001$, and $P < 0.0001$).

**Fig. 2.** Correlations of expression levels of six miRNAs in serum with the severity of liver fibrosis (sirius red staining) (a) and with the liver hydroxyproline content in S. japonicum-infected C57BL/6 and BALB/c mice (Pearson’s correlation coefficient).
and < 0.0001, respectively; for miR-200b-3p, $r = -0.4461, -0.4206$ and $-0.4408$, and $P = 0.0017, 0.0032$ and $0.0019$, respectively; for miR-146a-5p, $r = -0.5529, -0.4414$ and $-0.4927$, and $P < 0.0001, = 0.0019$ and $= 0.0004$, respectively). For miR-222, the inverse correlations were only observed in C57BL/6 mice ($r = -0.4258, -0.5036$ and $-0.5396$, and $P = 0.0028, = 0.0003$ and $< 0.0001$, respectively).

In C57BL/6 mice, the levels of miR-let-7a-5p and let-7d-5p in serum were inversely correlated with the intensities of hepatic fibrosis and granuloma (for let-7a-5p, $r = -0.3770$ and $-0.4224$, and $P = 0.0090$ and $0.0031$, respectively; for let-7d-5p, $r = -0.3160$ and $-0.3427$, and $P = 0.0305$ and $0.0184$, respectively). The serum level of miR-29a-3p positively correlated with liver HP content, the intensities of hepatic fibrosis and granuloma in BALB/c, but not in C57BL/6 mice. In BALB/c mice, the serum levels of miR-192a-5p and miR-29a-3p were positively correlated with the severity of liver necrosis ($r = 0.4517$ and $0.5085$, and $P = 0.0014$ and $0.0003$, respectively).

### 3.3. Correlations of the serum levels of six miRNAs with human schistosomiasis hepatic fibrosis

The expression levels of six miRNAs (miR-192a-5p, let-7a-5p, let-7d-5p, miR-185-5p, miRNA-29a-3p and miR-222) were inconsistent in the two mouse strains during infection with *S. japonicum*. This may have been due in part to the difference in hepatopathology (i.e., a higher level of liver necrosis was induced in BALB/c than in C57BL/6 mice at 6 weeks p.i. [6]), which likely fluctuate the levels of serum miRNAs. Therefore, C57BL/6 may represent a better model for exploring for potential circulating miRNA signatures in schistosomiasis liver fibrosis. Thus, six miRNAs (miR-150-5p, miR-200b-3p, let-7a-5p, let-7d-5p, miR-146a-5p and miR-222) dysregulated in C57BL/6 mice during the infection were selected for further validation with clinical samples. The potential association between the serum levels of the six miRNA signatures and fibrosis grades was then investigated in a human cohort from a schistosomiasis-endemic area of the Philippines (Table 2). The levels of four miRNAs (miR-150-5p, let-7a-5p, let-7d-5p and miR-146a-5p) in serum were inversely correlated with hepatic fibrosis grades ($r = -0.3218, -0.2732$, $-0.2371$ and $-0.2756$, and $P < 0.0001, = 0.0004, = 0.0023$ and $= 0.0004$, respectively) whereas the serum levels of miR-200b-3p and miR-222 did not significantly correlate with hepatic fibrosis grading (Fig. 3).

### 3.4. Discrimination of hepatic fibrosis grade 0-I and grade II-III by serum levels of six miRNAs

The expression levels of miRNA-150-5p, let-7a-5p, let-7d-5p and miRNA-146a-5p in serum were significantly lower in patients with fibrosis grade II-III than in those with grade 0-I ($P < 0.0001, = 0.0004, = 0.0027$ and $= 0.0029$, respectively), while the expression levels of miRNA-200-3p and miRNA-222 showed no significant difference between the two groups (Fig. 4a). The receiver operating characteristic curve analysis for discriminating liver fibrosis grade II-III to 0-I showed that the area under the curve (AUC) levels of miRNA-150-5p, let-7a-5p, and let-7d-5p were significantly lower in patients with fibrosis grade II-III than in those with grade 0-I ($P < 0.0001, = 0.0004, = 0.0027$ and $= 0.0029$, respectively). The area under the curve (AUC) levels of miRNA-200-3p and miRNA-222 did not significantly correlate with hepatic fibrosis grading (Fig. 4b).

### Table 2

*S. japonicum* infection status and intensity of 163 subjects at baseline stratified by fibrosis grade.

| Fibrosis grade | 0 (n = 52) | I (n = 35) | II (n = 47) | III (n = 29) |
|----------------|------------|------------|------------|-------------|
| M/F            | 23/29      | 24/11      | 37/10      | 28/1        |
| Age (years)    | 37.9 ±     | 34.94 ±    | 45.51 ±    | 50.52 ±     |
|                | 16.31      | 15.84      | 12.89      | 12.01       |
| Kato-Katz EPG  | (0–200)    | (0–650)    | (0–633)    | (0–2750)    |
| Negative (0)   | 42         | 21         | 23         | 14          |
| Mild (1–99)    | 9          | 6          | 20         | 11          |
| Moderate (100–399) | 1 | 6 | 3 | 2 |
| Heavy (>400)   | 0          | 2          | 1          | 2          |
| Serological test (+/-)* | 25 / 27 | 30 / 5 | 44 / 3 | 29 / 0 |

EPG: Egg per gram feces.

* Serological testing involved detection of IgG antibodies against a combination of *S. japonicum* recombinant antigens (SjSAP4 and Sj23-LHD) [6].

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### Fig. 3

Correlations of expression levels of six miRNAs in human serum with hepatic fibrosis grades (Spearman’s correlation coefficient).
let-7d-5p and miRNA-146a-5p were 0.6838, 0.6598, 0.6270 and 0.6575, respectively (*P < 0.0001, **P = 0.0004, ***P = 0.0052 and ****P = 0.0005, respectively) (Fig. 4b). No significant increase in AUC values was observed for the different combinations of the four circulating miRNAs in discriminating mild (grade 0-I) from severe (grade II-III) schistosomiasis liver fibrosis (Table 3). However, when the two extremes (grade 0 vs grade III) were compared, the AUC levels of miRNA-150-5p, let-7a-5p, let-7d-5p and miRNA-146a-5p were 0.7347, 0.7102, 0.6704 and 0.7172, respectively (*P = 0.0005, **P = 0.0018, 0.0114 and 0.0013, respectively), which were higher than those obtained in discriminating liver fibrosis grade II-III to 0-I (Supplementary Fig. 1).

3.5. Analysis of six miRNAs in serum in response to PZQ treatment in C57BL/6 mice post infection

C57BL/6 mice were used as a model for exploring the miRNA expression in serum before and after drug treatment. Fecal egg burden was assessed at 6 weeks p.i. in S. japonicum-infected C57BL/6 mice (Supplementary Fig. 2), and no significant difference was observed between the un-treated and treated (prior to treatment) groups. Considerable regression of liver fibrosis was observed in PZQ-treated mice at 6 months post-treatment (p.t.) shown by sirius red staining (Supplementary Fig. 3). The serum levels of miRNA-150-5p, miR-200b-3p, let-

Fig. 4. Discrimination of schistosomiasis mild (grade 0-I) and severe (grade II-III) hepatic fibrosis by expression levels of miRNAs in human serum. (a) Expression levels of the candidate miRNAs in serum of schistosomiasis japonica patients with (grade II-III) or without (grade 0-I) significant liver fibrosis. Boxes represent the interquartile range of the data with lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles, respectively. P values were determined using a Mann-Whitney U test (ns = no significant difference, *** = P < 0.01, **** = P < 0.001, ***** = P < 0.0001). (b) ROC curve analysis was performed and the AUCs were calculated to evaluate capabilities of serum miRNA levels for discriminating mild and severe schistosomiasis hepatic fibrosis.
7a-5p, let-7d-5p, miR-146a-5p and miR-222 were significantly downregulated at 6 weeks p.i. \( (P < 0.0001, < 0.0001, = 0.0006, = 0.0275 \text{ and } = 0.0265, \text{ respectively}) \); however, in the PZQ treatment group, the levels of these miRNAs were significantly increased at 6 months p.t. \( (P < 0.0001, < 0.0001, < 0.0001, = 0.0002, < 0.0001 \text{ and } = 0.0062, \text{ respectively}) \) (Fig. 5).

### 4. Discussion

The development of hepatopathology in schistosomiasis is a complex process, and mainly includes granuloma formation and the resultant development of liver fibrosis [10]. In addition to resident liver cells, immune cells recruited to the periphery of deposited eggs are also involved in the process, concomitant with a complicated regulatory interplay among these cell types. During these events, a complexed dysregulation of gene expression (including miRNAs) occurs in the infected liver tissue [4,7]. However, recent evidence has shown no direct correlation between the levels of miRNAs in the relevant tissues and in the circulatory system [35]. For example, no change of miR-150-5p or miR-200b-3p levels was observed in the liver tissue of BALB/c mice post *S. japonicum* infection [7], whereas we show here a down-regulation in the levels of these two miRNA in both infected C57BL/6 and BALB/c mice. This may be due to fact that miRNAs were expressed at different levels within multiple cell populations (resident cells and recruited cells) in the liver, and dysregulation of a specific miRNA usually occurred in particular cell types within the tissue. In addition, it has been argued that miRNA packaging into extracellular vesicles/exosomes is a selective process, although the precise regulatory mechanism for this remains elusive [16,23].

As one of the five miRNAs highly specific for hematopoietic cells, miR-150 is involved in the progression of renal and cardiac fibrosis [5,13,21,47]. Honda et al. [22] demonstrated the anti-fibrosis effect of miR-150 in fibroblasts by regulating the expression of integrin \( \beta 3, \)
been documented that miR-222 promotes the progression of liver fibrosis through regulating the activation of HSCs [36]. Although the serum levels of both miRNAs failed to discriminate mild from severe hepatic fibrosis, the expression of investigated serum miRNAs. Nevertheless, increased levels of miR-150-5p, let-7a-5p, let-7c-5p and miR-146a-5p were measured in the sera of C57BL/6 mice following the regression of liver fibrosis at 6 months post-treatment with PZQ, further indicating the potential utility of these miRNAs in staging schistosomal hepatic fibrosis.

The current work provides a key first step in identifying unique circulating host miRNAs associated with liver fibrosis caused by *S. japonicum* infection, only a limited number of miRNAs were tested in the study. In the future, it will be important to identify more potential miRNAs specific for schistosomiasis fibrosis based on serum miRNA profiling. Also, the relatively limited number of human samples tested may have impacted on our power to find statistically significant associations and increased diagnostic scores. A previous study showed that the use of vesicle-associated miRNAs produced a more sensitive representation of fibrosis grading [26], while another report did not [30]. It would thus be informative to determine whether serum vesicle-derived miRNAs improve on the ability in grading fibrosis in schistosomiasis.

In summary, by utilizing two mouse strains as schistosome infection models and then validation with a clinical cohort of schistosomiasis, we identified four circulating miRNAs that are associated with the staging of schistosomal hepatic fibrosis. The diagnostic performance of one of these miRNAs, miR-150-5p, for discriminating mild from severe fibrosis is comparable to the ELF test and serum level of HA. Circulating miRNAs may be a supplement for assessing hepatic fibrosis progression in human schistosomiasis.

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**Conflicts of interest**

All authors: No reported conflicts.

**Author contributions**

PFC and DPM conceptualized the study design and directed the project; PFC and YM performed all the experiments; RMO, AGR and DUO contributed to the collection of clinical samples; PFC, YM, and DPM analyzed, reviewed and interpreted the data; PFC drafted and DPM revised
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