Roles and applications of miRNAs in diagnosis, treatment, prognosis, and control of parasitic diseases. Part I: Helminthes

Review Article

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ABSTRACT

In the past two decades, the role of non-coding RNAs (ncRNAs) in inflammation gained much attention for their modification of inflammatory gene expression. They accordingly serve as diagnostic and/or prognostic biomarkers as well as potential therapeutic targets and vaccine candidates in various inflammatory diseases. These small (18-25 nucleotides) double-stranded ncRNA, microRNA (miRNA) are highly conserved occurring naturally in all eukaryotic genomes. They target specific messenger RNAs (mRNAs) to regulate their post-transcriptional expression resulting in translation suppression and gene silencing. Therefore, they act as master regulators of gene expression in homeostasis and disease. Proved evidences showed that all eukaryotes selectively sort miRNAs into extracellular vesicles (EVs) for secretion to nearby or distant targets. Besides, recent studies validated the ncRNAs role in RNA maturation, protein synthesis and post-translational pathway of autophagy. The aim of the present review is to understand ncRNAs functional roles with special emphasis on miRNAs and their potential applications in Parasitology research, as well as the effects of human miRNAs in host-parasite interactions.

Keywords: diagnostic biomarkers; drug targets; extracellular vesicles; helminths; miRNAs; prognostic assessment; vaccine candidates.

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INTRODUCTION

The ncRNAs play an important role in development, and several cellular processes by hybridizing with complementary target mRNA sequences of protein-coding transcripts, resulting in either mRNA cleavage, or inhibition of productive translation. According to length, localization, and functions, ncRNAs are either housekeeping or regulatory. While housekeeper ncRNAs include nuclear (nRNAs), transfer (tRNAs), and ribosomal (rRNAs), those involved in regulation of the gene expression include miRNAs, long noncoding (lncRNAs), circular (circRNAs), small interfering (siRNAs), and PIWI-interacting (piRNAs). The *piwi* genes, the largest class of ncRNAs, are regulatory proteins for stem and germ cells differentiation. Notably, PIWI proteins belong to the Argonaute/Piwi family responsible for epigenetic regulation, and post-transcriptional silencing. They also contribute in maturation of housekeeping RNAs. In fact, ncRNAs are major epigenetic tools of transcriptional and post-transcriptional aspects of gene expression that work on regulatory proteins as activators, or repressors, or chromatin modulators[1].

Variable environmental conditions such as change in temperature, and nutrients availability induced challenges to the pathogen development that required change in gene expression. In fact, miRNAs have essential effects on the cellular transcriptions by two main diverse mechanisms. They either promote cell proliferation through inhibition of cell cycle arrest pathways, or inhibit differentiation factors that prevent premature differentiation. They also utilize two other mechanisms, either downregulation of differentiation to elicit broad transcriptomic changes, or restrict cell fate. Whatever the mechanism involved, miRNAs have a significant capacity to regulate the gene expression at the post-transcriptional level in a sequence-specific approach. This involves translation suppression or mRNA degradation to achieve fine-tuning biological impact. Timekeeping is a repeated theme in miRNAs biology, i.e., miRNAs orchestrate gene expression to maintain biological rhythm and dynamics during cell development. They perform as switch pacemakers that turn on or off to specify cellular transcription at specific times. Determination of individual miRNA and its mRNA target would certainly facilitate mapping miRNA-mRNA interactome, i.e., interactions between intracellular molecules either within a cell or within the whole organism[2].

Similar to miRNAs, lncRNAs (>200 nucleotides) have a diverse genomic location, their sequence is very little-conserved among species, and produced by all tissues, but is abundant in the brain and central nervous system. Their ability to bind to DNA, other RNAs, and proteins allowed them to serve as signaling molecules, and scaffolds for DNA-binding proteins. Besides, they are able to change the patterns of chromatin

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organization, therefore they participate in miRNAs modification, activate or repress gene activation, and can act as competitive endogenous RNAs (ceRNAs). Notably, ceRNAs are lncRNAs with efficient ability to compete miRNAs regulation in abundance of other miRNA targets. Due to insufficient studies conducted on lncRNAs interactions, mechanisms of action, and functions, further studies to better understand lncRNAs roles as gene expression regulators, and epigenetic molecules were recommended[3].

Interestingly, miRNAs were originally discovered while screening genes of mutant free-living *Caenorhabditis elegans* that were unable to control the timing of specific cell fate switching during development. In the present review, we aim to demonstrate our current understanding of the molecular biological influence of miRNAs in the development and progression of parasitic diseases. It is hypothesized that identification and characterization of parasite-specific miRNAs and their targets, as well as host-related miRNAs would certainly help identify diagnostic, and prognostic biomarkers, as well as novel therapeutic and protective agents to control major neglected tropical diseases.

Biogenesis

The most widely used route for miRNAs biogenesis is the canonical pathway that consists of several steps. It starts in the cell nucleus where genes for miRNA are transcribed as part of RNA polymerase II dependent transcripts, and fold into long double-strand primary miRNA transcripts (pri-miRNA). A microprocessor complex formed by ribonuclease III enzyme (RNase) and Drosha-Pasha complex, processes the pri-miRNA to form miRNA precursor (pre-miRNA) composed of 70-100 nucleotides. To protect the pre-miRNAs from nuclease degradation, it is translocated to cell cytoplasm using exportin 5/guanine triphosphatase (GTPase) complex. In the cytoplasm, the pre-miRNA undergoes cleavage by another complex that consists of RNase III Dicer, and transactivation responsive RNA-binding protein to remove the terminal loop, resulting in a ~22 nucleotides mature miRNA duplex. Then, the duplex is separated by a helicase, and the single miRNA strand is incorporated into the RNA-induced silencing complex (RISC) and coupled with argonaute protein family that guides RISC to interact with the target mRNA[4].

Because miRNAs interact with the 3′ untranslated region (3′-UTR), and 5′-UTR of the target mRNA, such partial or full complementary binding (interaction) leads to regulation of gene expression either by mRNA degradation or inhibiting its translation. The simultaneous ability of miRNAs interaction confirms its involvement in disease pathogenesis, i.e., its ability to interact with diverse mRNAs facilitates control of gene expression involved in the inflammatory response according to the induction of host immune response. The miRNA:mRNA target interaction suggests that the sequence context of the miRNA binding site is critical for its function. To achieve their function as gene regulatory agents, a combined control within either the cell or cell-to-cell variation was suggested. In such control, miRNA sequence is either complementary to only one mRNA, or near complementary to multiple mRNAs encoded by distinctly different genes. Therefore, a single miRNA may bind with a single mRNA or multiple mRNAs. Similarly, a single or multiple mRNA(s) is (are) target(s) for multiple miRNAs, In cell-cell variation, switching on/off of the transcript is the main functional role of miRNAs to control and maintain cellular differentiation that requires multiple gene expression[1].

Human miRNAs

In human, thousands of genes encoding miRNAs are identified, and 20-30% of mRNAs are targeted by miRNA. During the last two decades, they attracted much attention as candidate drug targets for cancer, metabolic diseases, and viral infections due to their implication in regulation of genomic rearrangements associated with carcinogenesis, insulin secretion, and resistance to viral infection, respectively. Nowadays, research in ncRNAs enables scientists and clinicians to discover molecules that help to modify diagnosis, differentiate between malignant and benign tissues, diagnose early cancer, and predict its prognosis. Besides, development of diagnostic and prognostic biomarkers that are detected in urine or blood may serve as an ideal diagnostic approach to avoid invasive procedures associated usually with histopathological examination. Moreover, identification of certain ncRNAs can determine risk of metastasis, and chemotherapeutic response, in addition to their usefulness in development of novel drugs in oncology medicine[5]. Conversely, plasma miRNAs circulate in four different molecular forms. The first is released in vesicles (100–1000 nm) produced from the cell surface by budding of the outer cell membrane. The second is released in exosomes (50-100 nm) produced by multivesicular bodies and contain DNA, mRNA, proteins, and miRNAs. The third is the apoptotic bodies (1–5 μm) produced in the apoptotic process. The fourth is a protein or lipid-bound form including argonaute2 (Ago2) and high-density lipoprotein. All forms are stable in circulation, especially exosome-included miRNAs. Their levels in serum, saliva, and urine are lower than in plasma[6].

A remarkable human miRNA property is that it has isomers. Next-generation sequencing studies showed that diverse variants of a single miRNA (isomiRNAs) can affect the miRNA target selection. There are also several aspects of human miRNA interactions such as the interaction between epigenetics and miRNA in which 1) different miRNAs are epigenetically regulated by multiple DNA methylation enzymes, 2) DNA methylation is controlled by diverse mechanisms

related to miRNAs, 3) a direct and indirect crosstalk between miRNA and lncRNAs, such as competing endogenous RNA, controls miRNA-mRNA binding to conduct intercellular regulation that is involved in the pathogenesis of different diseases, 4) an interaction of miRNAs and factors released during viral, bacterial, and parasitic infections leads to modulation of disease pathogenesis. Therefore, the up- and down-regulation of specific human miRNAs can control pathogens virulence genes. The reviewers concluded reciprocal associations between host and pathogens miRNAs, i.e., ability of human cellular miRNA networks as a tool to control infections dissemination, and pathogen-derived miRNAs that target host's transcripts related to immune response[7].

In a recent report, Tamgue *et al*.[8] reviewed the studies conducted on human miRNAs and lncRNAs, and concluded that they are emerging key regulators of gene expression in immune cells differentiation, activation, and function, including macrophages, dendritic cells, and T lymphocytes. The reviewers summarized the role of ncRNAs in the etiology and control of major tropical diseases including tuberculosis, HIV/AIDS, malaria, leishmaniasis, African trypanosomiasis and leprosy. They also highlighted several ncRNAs as potential diagnostic and prognostic biomarkers since they are involved at different stages of disease development. In addition, other ncRNAs emerged as potential novel therapeutic and protective targets[8].

The fast evolution in molecular microarray technology enabled scientists to sequence human miRNAs regulating cellular response that result in signaling and pathophysiological modifications during pathogenic infections. Besides, host miRNAs are also involved in mediating intercellular communications since they are secreted into vesicles that circulate in extracellular fluids as exosomes, i.e., they present potential diagnostic and prognostic biomarkers for a variety of disorders, including parasitic diseases[9]. There are several developed databases for miRNAs that provided nomenclature for all published miRNAs from human and several pathogens, miRNA genes, and their sequences. Online libraries including miRbase (www.mirbase.org), MirGeneDB 2.0 (mirgenedb.org/), MiRCarta (mircarta.cs.uni-saarland.de/), and TargetScan (www.targetscan.org/vert_72/) were established to describe miRNAs involvement in several complex regulatory networks to understand, on molecular level, the pathophysiological aspects of several diseases[7].

Roles of miRNAs in host-parasite interactions

In 2022, Rojas-Pirela and his colleagues[10,11] published two reports demonstrating that dysregulation of miRNAs biogenesis and function are directly related to the pathogenesis of several parasite infections caused by either protozoans[10], or helminths[11]. Both parasite and host miRNAs contribute in host-parasite interactions determining infection probability and diseases progress. The reviewers claimed that intracellular miRNAs are located in 1) cytoplasm where post-transcriptional silencing is the classic function mediated by miRNA *via* RISC, 2) nucleus to regulate the functions of nuclear miRNAs, 3) mitochondria targeting mitochondrial mRNAs, 4) endoplasmic reticulum to repress the translation that occurs in the membrane-bound polysomes, 5) Golgi apparatus where they are involved in resistance to chemotherapeutic drugs in cancer cells, 6) EVs where they essentially contribute in cell-to-cell communication, and 6) the processing bodies where they are necessary for their formation and integrity. The latter are cytoplasmic ribonucleoprotein granules with specific roles in post-transcriptional regulation that are dynamically formed during the cell cycle in response to extracellular signals. It was concluded that during host-parasite interactions, parasite miRNAs mediate post-transcriptional regulation of genes involved in disease pathogenesis, and modulate host miRNAs involved in the cellular immune response against parasite replication[10].

In another review, Mexican scientists[3] discussed the role of parasitic miRNAs, and lncRNAs in the etiopathogenesis of cancers commonly reported in association with flukes; *S. haematobium, C. sinensis,* and *O. viverrine*. The reviewers proposed different processes that may lead to chronic inflammation, polarization of immune cells, e.g., macrophages and T cells, severe induction of tissue persistent injury, and tumorigenesis. In certain parasitic infections, specific ncRNAs are highly expressed regulating the previously mentioned processes. Therefore, detection of parasitic ncRNAs in blood or urine was considered a potential diagnostic biomarker for tumorigenesis[3].

Since parasite-derived miRNAs are a characteristic component of EVs circulating in body fluids (serum, saliva, and urine), and tissues of the infected host, they are considered stable potential non-invasive diagnostic biomarkers. Recently, a review discussed the potential use of extracellular/circulating helminth-derived miRNAs for early-stage detection of helminth infection, i.e., regarded as non-invasive clinical samples. The reviewers claimed that extracellular helminth-derived miRNAs predominantly exist in exosomes in serum and saliva of the infected host. It was hypothesized that their high stability under adverse conditions is attributed to their small size, establishment of a miRNA-protein complex, and their fusion into exosomes. Although the majority of helminth-derived miRNAs are endogenous, only few are able to enter host circulation to be detected in blood plasma, serum, and urine. Several sensitive methods were utilized for their detection and identification such as Northern blot, *in situ* hybridization, real-time PCR, miRNA microarray and next-generation sequencing. Certain criteria were provided for

accurate diagnosis including storage conditions, correct selection of primers, and professional experience. However, their use as potential diagnostic and/or prognostic biomarkers have essential limitations such as expensive instrumentation, the need for well-equipped laboratories, and cross-reactivity[12].

It is worth mentioning that environmental changes, e.g., global warming, contribute to keep vector-borne diseases a worldwide public health concern. In vector-borne diseases, several miRNAs are incriminated and interact with multiple target genes to elicit essential biological functions favoring life cycle stages metabolism, survival, growth, and differentiation. Although characterization and function of these miRNAs and their potential targets have not been fully determined, Chinese reviewers summarized several studies conducted in mosquitoes to understand miRNA's role(s) on their variability in transmission efficiency, and their susceptibility to infection, i.e., vector-pathogen interactions. Understanding the molecular and genetic mechanisms of transmission dynamics certainly help in development of a novel vector control strategy. The reviewers tabulated hundreds of putative miRNAs identified in different *Aedes, Culex,* and *Anopheles* spp., and categorized the identified miRNAs according to their functions, and vector life cycle stages. They also drew a diagram showing the differentially regulated miRNAs (up or down regulation) according to the infection (malaria, Dengue fever, and West Nile fever), as well as according to their susceptibility or resistance to pyrethroid. The reviewers observed species-, stage-, sex-, and tissue/organ-specific miRNAs, and the most commonly reported miRNAs were miR-281, miR-184, miR-989, and miR-278[13].

Applications of miRNA in parasitic diseases caused by helminths

Schistosomiasis

Schistosomes life cycle complexity (seven discrete developmental stages) requires dynamic morphological and transcriptional changes that occur within both hosts. This reflects *Schistosoma* needs to adapt to constant changes during its growth, differentiation, and development that are partially regulated by several miRNAs.

Survival and differentiation: In a recent study, Zhou *et al*.[14] observed that *S. japonicum* miRNAs exhibited different expression patterns of miR-124-3p at different developmental stages being significantly higher in cercaria than in schistosomula, in juveniles than in adults, in males than in females, and in immature females than in mature ones. Using reverse transcriptase quantitative PCR (RT-qPCR), the study demonstrated that *Sj*-miR-124-3p showed significant expression levels that differed according to the hosts (resistant, unsuitable, susceptible), developmental stages, and schistosome' gender. The investigators attributed this phenomenon to differences in the metabolic demand of each stage for *Sj*-miR-124-3p gene expression to survive and grow. Using dual-luciferase reporter assay system, the investigators identified a potential target gene; ATP-dependent RNA helicase DDX1 (*Sj*DDX1). In mice injected with *Sj*-miR-124-3p-agomir (a chemically modified small RNA that mimics endogenous miRNA), the investigators conducted RNA interference of the gene encoding *Sj*DDX1 showing damaged adults, and reduced hepatic pathological changes due to altered growth, development, and oviposition of *S. japonicum*. It was hypothesized that *Sj*-miR-124-3p balanced expression by different developmental stages was required for development and reproduction through regulation of neurosecretory cells to express essential molecules for survival. Besides, upregulation of *Sj*-miR-124-3p inhibits the secretion of schistosomes antioxidant enzymes affecting their growth and development, i.e., switch on/off dynamic regulation[14].

Several studies were conducted to investigate changes in miRNAs expression regulating sexual differentiation of the three main *Schistosoma* spp. In *S. japonicum*, 14 miRNAs were enriched in males, among them *Sj*-miR-7, *Sj*-miR-61, and *Sj*-miR-219, and four in females, including *Sj*-bantam and *Sj*-miR-31 predominantly localized to the ovary[15]. Two-thirds of the identified miRNAs in 90-day *S. haematobium* adults exhibited substantial female-biased transcription including *Sh*-miR-71a, *Sh*-miR-71b, *Sh*-miR-2162, and *Sh*-bantam. Only 5% were sex-biased in males that included *Sh*-let-7, *Sh*-miR-1, *Sh*-miR-7a, and *Sh*-miR-125b[16]. Three years later, a Brazilian study observed enrichment of *S. mansoni* couples with *Sm*-miR-92a, *Sm*-miR-250, and *Sm*-miR-5-5p[17]. In the last study, using RT-qPCR, the investigators assessed 12 *S. mansoni* miRNAs to identify their putative mRNA targets during three developmental stages (cercaria, early schistosomula, and adult). Differential expression patterns were recorded for eight of them (miR-250, miR-92a, miR-4-3p, miR-4-5p, miR-5-5p, miR-12-5p, miR-13-3p, and miR-13-5p). They were up-regulated in adults, and their putative target genes were linked mainly to oxidative phosphorylation, i.e., post-transcriptional mitochondrial regulation. These genes were related to three issues: 1) energetic glucose and lipid metabolism, 2) transforming growth factor-β (TGF-β) signaling pathway, and 3) immune system evasion. Moreover, main seven mRNAs were identified target genes for the investigated miRNAs including three mitochondrial NADH ubiquinone oxidoreductase subunits, cytochrome C type heme lyase, ATP synthase lipid-binding protein-like protein, ubiquinone biosynthesis protein, and mitochondrial carrier 2 protein. The investigators drew a diagrammatic figure to demonstrate putative *S. mansoni* miRNAs involved in the regulation of oxidative phosphorylation during its developmental stages[17]. From these studies[15-17], it was concluded that the putative target genes of stage-specific and gender-biased miRNAs in schistosomes

were mostly linked to TGF-β, and chemokine signaling pathways, and both are involved in schistosomes development and embryogenesis. Recently, 225, and 79 miRNAs, identified in miRBase (https://mirbase.org/), were recognized upregulated in different developmental stages of *S. mansoni*, and *S. japonicum*, respectively. The reviewers tabulated these miRNAs according to their roles in host-parasite interactions; cell-cell communication through EVs, hepatic fibrosis, influence on host miRNAs[18].

Host immunomodulation and infection establishment: It was documented that *Schistosoma* EVs with their cargo miRNAs enhanced TNF-α production[11], host macrophages and monocytes proliferation[19], and downregulated nuclear factor (NF-kβ) pathway[20] inducing immunomodulation of host gene expression involved in host immune response to facilitate parasitism. During infection, miR-10, miR-125, and bantam of *S. mansoni* EVs were released into T lymphocytes modulating the signaling pathways to negatively regulate gene expression through NF-kβ activation. The latter diminishes T cell differentiation into Th2 subpopulations. Whereas during *S. japonicum* infection, EVs *Sj*-miR-125b, and bantam were released in macrophages to 1) increases their proliferation, 2) inhibit toll like receptors (TLRs)-mediated inflammation, 3) stimulate TNF-α production and release into extracellular environment for *S. japonicum* development, survival, metabolism, and egg deposition[11].

Hepatic fibrosis: In their review, Chen and his colleagues[21] discussed *Schistosoma* miRNA roles in the pathogenesis, grading, and treatment of hepatic fibrosis. It was claimed that hepatic fibrosis, i.e., excessive deposition of extracellular matrix, occurs *via* multiple signaling pathways triggering the activation of hepatic stellate cells (HSCs), Kupffer cells, and liver sinusoidal endothelial cells. Accordingly, the reviewers tabulated variable miRNAs involved in the pathogenesis of hepatic fibrosis either with pro-fibrogenic or anti-fibrogenic roles. The pro-fibrogenic miRNAs included miR-27b (enhancing HSCs activation), and miR-21, miR-96 and miR-351 (activating SMAD signaling pathway). On the other hand, several miRNAs were involved with anti-fibrogenic roles; miR-203-3p (inhibiting IL-33 secretion), Let-7 [targeting TGF-β transmembrane receptor-1 (TβR1)], miR-15b and miR-16 (inducing HSCs apoptosis through caspase signaling pathway), miR-454, miR-155 and miR-29b-3p (inhibiting HSCs activation), and miR-146a/b (regulating macrophages transformation from M1 to M2/IFN-γ signaling pathway)[21]. In another recent report[18], the reviewers discussed miRNAs mechanisms involved in induction of hepatic fibrosis. First, schistosome-derived miRNAs induced HSCs differentiation into collagen-producing myofibroblasts. They also activated multiple signaling pathways [(TβR1, SMADs, and phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)] through alteration of host miRNAs, leading to HSCs activation, and increased hepatic fibrosis. It is worth mentioning that SMADs are intracellular proteins that translocate TGF-β from the cytoplasm to the nucleus to control gene expression. Besides, PI3K/AKT signaling pathway is an intracellular signal transduction pathway promoting metabolism, proliferation, cell survival, growth and angiogenesis in response to extracellular signals. Second, alteration of host miRNA expression, involved in immune response, participates in regulation of the pathogenesis of hepatic fibrosis. During early phase of schistosomiasis *japonicum*, there was altered host miRNAs expression related to mitogen-activated protein kinase (MAPK), TLRs, and TGF-β signaling pathways. Third, schistosome miRNAs mediate cross-species host-parasite interaction through EVs. Therefore, circulating and EV-bound miRNAs are potential diagnostic and prognostic grading markers of hepatic fibrosis; discussed later[18].

Diagnostic and prognostic markers: *Schistosoma* miRNAs (*Sj*-miR-277 and *Sj*-miR-3479-3p) proved potential biomarkers for grading hepatic fibrosis[22]. Host miR-223 was significantly upregulated in *S. japonicum*-infected mice[23] that returned to normal level after Praziquantel (PZQ) treatment[24]. Combined use of several host miRNAs was suggested to evaluate severity of hepatic fibrosis in murine schistosomiasis[22]. Bantam and miR-2c-3p isolated from serum extracellular vesicles of infected patients were suggested as follow-up biomarkers[25]. In 2018, two studies were conducted when the investigators proposed four circulating miRNAs (miR-150-5p, let-7a-5p, let-7d-5p and miR-146a-5p) to discriminate between mild and severe hepatic fibrosis in patients with schistosomiasis, and miR-150-5p displayed the best diagnostic performance for grading hepatic fibrosis[26]. The second study demonstrated that *S. mansoni* released exosome-like vesicles *in vitro* containing 143 miRNAs, of which *Sm*-miR125b, and *Sm*-bantam were expressed at significant high levels. Using real-time qPCR analysis, the investigators succeeded to identify both schistosome-derived miRNAs in exosomes purified from sera collected from experimentally infected mice. The study recommended further studies to validate *Sm*-miR125b, and *Sm*-bantam potential diagnostic biomarkers[27].

To investigate the correlation between progress of hepatic fibrosis in schistosomiasis and exosome-derived miRNAs, the investigators carried out a prior study to select validated stable serum miRNAs. The pilot study investigated the expression of 12 serum miRNAs in murine schistosomiasis, and 104 patients with different grades of *S. japonicum*-associated liver fibrosis. Using qRT-PCR, the study determined the expression profiles of 9 fibrosis-associated miRNAs in C57BL/6 mice during infection. The investigators observed that combined use of miR-146a-5p with miR-532-5p had the best performance to diagnose

hepatic fibrosis. In spite of its insignificance, miR-146a-5p was recorded as a potential prognostic biomarker differentiating between with mild (grades 0–I) and severe fibrosis (grades II–III)[28]. Recently, Egyptian investigators studied host miR-223 and miR-146b expressions in relation to egg deposition, and development of hepatic fibrosis in *S. mansoni* infected mice. Blood samples, collected from non-infected and infected mice at different time intervals, and four weeks after PZQ treatment, were subjected to RNA extraction, reverse transcription, and real-time PCR assays. Results of host miR-223 and miR-146b expressions were correlated with hepatic egg counts and histopathological fibrosis at the same time intervals. The investigators recorded significant miR-223 downregulation at 8 and 12 weeks, with gradual increase of miR-146b expression reaching a significantly higher level at the 12th week. These results were significantly correlated with hepatic egg burden, and more observed in distorted hepatic architecture at the 12th week. Interestingly, both host miRNAs restored their normal expression levels after PZQ treatment. Accordingly, the study suggested using miR-223 and miR-146b potential prognostic biomarkers for hepatic fibrosis induced by schistosomiasis *mansoni*[29].

Additionally, Ullah and his colleagues[12] tabulated and summarized 20 circulating *S. japonicum* miRNAs that were upregulated in several hosts including human, mouse, rabbit, and buffalo. In human infections with *S. mansoni*, and experimentally-infected mice, only three miRNAs were recorded. The reviewers also tabulated and discussed the roles of *Schistosoma* miRNAs cargo in EVs in different developmental stages[12].

Carcinogenesis: A recent review claimed that the five top-ranked *S. haematobium* miRNAs were miR-1, miR-71a, miR-125b, miR-7a, and miR-let-7[3]. Among them, miR-71 was proposed by an Egyptian study[30] as a potential biomarker to predict cancer bladder. Expression of *S. haematobium* miR-71 and MAPK-3 in urine samples of patients with bladder cancer or patients with benign bilharzial cystitis were significantly higher than those recorded in the urine of patients with non-bilharzial bladder cancer. Both non-invasive biomarkers showed significant positive correlation with malignancy grade[30]. In contrast, a recent review denied *Sj*-miRNAs role in hepatocellular and colorectal carcinoma associated with schistosomiasis *japonicum*. The reviewer attributed cancer to chronic inflammation, and DNA damage[31].

Therapeutic role: Delivering miRNA antagonists or mimics to compete with endogenous RNAs provided a promising strategy to treat schistosomiasis-associated liver fibrosis. Since they may exhibit unwanted side effects, their delivery *via* adeno-associated virus, i.e., vector-based miRNA inhibition showed efficient results. Inhibition of miR-96[32] or miR-21[33] or miR-203-3p[34] using recombinant adeno-associated virus serotype 8 (rAAV8)-mediated delivery effectively ameliorated hepatic fibrosis with significant decreased expression of TβR1, IL-4 and IFN-γ, and reduced collagen I and III.

Recently, a study observed that *Sm*-miR-10 influenced host T-cells that immunomodulate immune response through downregulation of NF-κB pathway. Using a fragment-based screening approach, the investigators succeeded to identify a *Sm*-miR-10 suppressor to prevent its processing and maturation in EVs. The investigators hypothesized that such potential immunotherapy enhanced sustained Th2 response after the initial 8-week phase to clear the infection[20]. Another recent study conducted in China identified and characterized miR-33, a novel egg-derived exosome miRNA, that upregulated the expression of smooth muscle actin (α-SMA), and collagen 1-α1 in HSCs cell line *in vitro*, and *in vivo*. The study demonstrated that *Sj*-miR-33 activated HSCs, and upregulated TβR1) are involved in TGF-β/SMAD3 signaling pathway to promote hepatic fibrosis. The degree of hepatic fibrosis in experimentally-infected mice was significantly reduced after treatment with miRNA antagomir. Such antagomirs are a class of chemically engineered oligonucleotides designed to silence endogenous miRNAs (anti-miRNA). The investigators hypothesized that *Sj*-miR-33 can regulate the post-transcriptional process of the host in a cross-species manner, and proposed it a potential drug target in ameliorating severe hepatic fibrosis caused by *S. japonicum*[35].

Anti-cancer therapy: Several Chinese studies utilized the approach of "cross-species regulation of genes suppressing cancer" to demonstrate the value of *S. japonicum* miRNA as potential anti-cancer therapy. It is worth mentioning that human miR-7-5p is established as a tumor suppressor miRNA that inhibits tumor growth by regulating multiple oncogenic signal pathways. Accordingly, a study hypothesized that *Sj*-miR-7-5p, with similar molecular structure with its human orthologue may induce cell cycle arrest inhibiting tumor growth *in vivo*. To achieve this, the investigators transfected, *in vitro*, human hepatoma cell lines with *Sj*-miR-7-5p isolated from infected hepatocytes. They observed that tumor size was significantly reduced when a xenograft mice model was inoculated with the transfect culture. The study attributed their results to the proposed hypothesis that *Sj*-miR-7-5p may increase host resistance to hepatocellular carcinoma during infection[36]. Using a similar approach, the same group of investigators characterized *Sj*-miR-61 with anti-angiogenesis properties and suppression of cell migration by down-regulation of phosphoglycerate mutase 1 (PGAM1) expression. The study demonstrated that host *pgam1* was a target gene for *Sj*-miR-61[37]. A third study demonstrated that *Sj*-miR-3096, carried by EVs released by *S. japonicum* eggs, exhibited *in vitro*, and *in*

vivo antitumor effects, and suppressed cell proliferation of human hepatoma cell lines. The investigators attributed their results to the cross-species regulation of the gene encoding PI3K. Notably, PI3K pathway is involved in cancer mutagenesis and progression *via* modulation of mammalian target of rapamycin complex 1 (mTORC1) that have an essential role in activation of proteins translation[38]. Through screening 57 *S. japonicum* miRNAs with antitumor activity *in vitro* and *in vivo* models, Frizzled class receptor 4 (*fzd4* gene), involved in cancer promotion, was also demonstrated as *Sj*-miR-71a target gene[39].

Fascioliasis

Survival and growth: Using an integrated sequencing, bioinformatics analyses, and real-time qPCR, miRNAs target genes differ among *Fasciola* spp. It was observed that predicted target genes in *F. gigantica* were mostly transcriptional regulators, while in *F. hepatica*, they were proteins related to development, reproduction, and locomotion. It was attributed to the differences of their intermediate hosts, and metabolic adaptations[40]. In both species, miRNAs play essential roles in the migration of newly encysted juveniles (NEJs) during their tissue invasion[40]. Besides, miR-277 expression was reported to be involved in enzymes regulation related to catabolism of aliphatic amino acids, with essential roles in the survival under stress conditions of starvation[42].

Immunomodulation of host immune response: Ricafrente and her Australian colleagues[43] observed that by inhibition of the immediate host protective immune response, *F. hepatica* ensures survival of the NEJs to pass across the peritoneal cavity reaching their habitat. In the liver, the host response switches to a Th2 phenotype mediating hepatic tissue repair. Accordingly, they hypothesized that continued characterization and functional analysis of *F. hepatica* miRNAs would reveal the molecular biological regulation of immune cell pathways with enormous benefit to the development of novel strategies for infection control. They collected the conducted researches that identified miRNAs in NEJs and adults, and using their sequences to predict their host gene targets. To achieve correct annotation, they compared published sequences to those recorded in miRBase for *F. hepatica* mature miRNAs. Interestingly, the majority of the identified 77 miRNAs in *F. hepatica* miRnome targeted host innate, and adaptive immune signaling pathways, i.e., with high potentiality to target genes regulating dendritic cells, eosinophils, macrophages, and neutrophils activation[43].

Infection establishment: *F. hepatica* miR-125b, miR-2b-A, miR-2a-B, miR-87, and miR-1993 were the most highly expressed in EVs containing miRNAs cargo. Among them was miR-125b that shared sequence identity with mammalian miRNAs, hijacked host homologue and immunomodulated host macrophage miRNA machinery pathway decreasing inflammatory cytokines through inhibition of MAPK-3 signaling pathway[44].

Diagnostic biomarkers: A Chinese study identified two *F. gigantica* miRNAs (*Fg*-miR-87, and *Fg*-miR-71) specifically circulating in the sera of 121 infected buffaloes, and proposed them diagnostic biomarkers[45].

Clonorchiasis and Opisthorchiasis

Conserved miRNA sequences: Using deep sequencing-bioinformatic approach, a Russian study identified 19 conserved miRNAs among members of the family Opisthorchiidae. While 18 miRNAs were conserved for each of *O. felineus* and *C. sinensis, O. viverrini* had 16 conserved miRNAs. There were differences in the expression level of conserved miRNAs among the oriental hepatic flukes. Identification and characterization of these miRNA genes would facilitate development of novel approaches to control infections[46].

Immunomodulation of host immune response: In a recent Chinese study, the investigators demonstrated enrichment of let-7a-5p in *C. sinensis* EVs that facilitated M1-like macrophages activation with induction of severe biliary injuries. Besides, the study showed *Cs*-let-7a-5p mechanism of action which was through increasing proinflammatory responses *via* targeting NF-κB signaling pathway[47].

Hepatic fibrosis: Utilizing miRNA microarray, and real-time qPCR, the expression profiles of miRNAs from *C. sinensis*-infected mice at different time intervals (2, 8, and 16 w) were analyzed. Several miRNAs were differentially expressed, and potentially involved in the pathological processes of clonorchiasis. The study demonstrated that the majority of *Cs*-miRNAs targeted host genes regulating signaling pathways involved in biliary fibrosis, was previously described in schistosomiasis-associated hepatic fibrosis such as TGF-β/ SMAD, MAPK-3, PI3K/AKT, and TLRs. Among them, miR-497 was highly expressed and negatively correlated with SMAD7 expression, i.e., it down-regulated SMAD7 expression with increase of biliary fibrosis. The investigators proposed miR-497 inhibitors potential novel therapeutic agents for decreasing hepatic fibrosis[48]. Later, the same group of investigators demonstrated target genes of *Cs*-miR-497. First, human hepatic stellate LX-2 cells were used to investigate their roles and mechanism of action to promote hepatic fibrosis *in vitro*. The LX-2 cells transfected with miR-497 inhibitor were stimulated with TGF-β1 or *C. sinensis* excretory/ secretory products (ESPs), and LX-2 activation was assessed using RT-qPCR. Although LX-2 transfection, miR-497 overexpression was recorded by stimulation with either TGF-β1 or ESPs. Second, to establish a liver fibrosis mouse model, mice were treated with a chemokine ligand (CCl4) injection for 6 w, then

intravenously injected with a single dose of adeno-associated virus serotype 8 (AAV8) that overexpressed anti-miR-497 sequences. Using combined bioinformatics analysis with luciferase activity assays, the study elucidated miR-497 mechanism of action through activation of TGF-β/SMAD signaling pathway by targeting *smad7* gene. The investigators concluded that *Cs*-miR-497 promoted LX-2 activation *in vitro*, and exacerbated liver fibrosis *in vivo*[49].

Diagnostic biomarker: Two miRNAs (*Ov*-miR-76 and *Ov*-miR-new1) showed different sequences that could be used in differentiating between *O. viverrini* and other related hepatic flukes, *O. felineus* or *C. sinensis*[50].

Carcinogenic biomarkers: Several studies demonstrated the involvement of host circulating miRNAs in cell differentiation/proliferation, inflammation, metastasis, and oncogenesis through regulation of various cancer-related signaling pathways. Functional clustering of these dysregulated host miRNAs revealed their role in TGF-β, MAPK, TLR, and PI3K/AKT signaling pathways as well as by targeting several genes encoding matrix metalloproteinase-9 (MMP9), tyrosine-protein phosphatase, and serine/threonine protein kinase 1[48,51-53]. Using pooled serum collected from patients with *O. viverrini*-associated cholangiocarcinoma (CCA), a Thailand study demonstrated significant higher levels of circulating miR-192 in CCA patients' serum than in healthy donors. Compared to individuals with low serum miR-192 level, high miR-192 levels were significantly correlated with lymph nodes metastasis, and shorter survival rate. The investigators proposed host circulating miR-192 a non-invasive prognostic biomarker for CCA patients[52]. Using small RNA-Seq and real-time qPCR on matched plasma samples from patients with intrahepatic CCA, American investigators profiled expression levels of host circulating miRNAs in CCA tumor tissue. Results revealed increased expression levels of eight miRNAs to the extent that normal tissue adjacent to CCA sowed significant high levels, i.e., similar to tumor than liver tissue from healthy donors. The study suggested using the eight-miRNA signature for early detection of CCA[53].

Hydatid cyst

Echinococcus genomes have ~10-14% protein-coding genes, while the remaining genes are transcribed as ncRNAs. The expression profile of *Echinococcus* miRNAs is species- and developmental stage-specific. Several common miRNAs were identified in three *Echinococcus* spp. affecting man (*E. granulosus sensu lato*, *E. multilocularis*, and *E. canadensis*), and their intermediate hosts. Chinese reviewers[54] tabulated 76 *E. granulosus* and 46 for each of *E. multilocularis*, and *E. canadensis* that were reported in 20 publications from 2011-2019. Their transcriptome analyses revealed ≥87% identity between *E. granulosus* and *E. multilocularis* miRNAs. While miR-2, miR-71, and miR-125 had the highest expression levels in different *E. granulosus* developmental stages, miR-71, and let-7 were the most abundantly expressed in one stage (hydatid cyst) of *E. canadensis*. In *E. multilocularis*, five miRNAs (let-7, miR-10, bantam, miR-71, and miR-9) were the most highly expressed miRNAs. Several miRNAs were specifically expressed in the cyst walls of secondary hydatid cyst and proved stage-specific. The reviewers also tabulated 19 commonly reported miRNAs and described their biological functions according to the identified target genes[54].

Immunomodulation of host immune response: Several studies were conducted elucidating the functional roles of *E. multilocularis* miRNAs to immunomodulate host immune response. Among them, *Em*-miR-14-3-3, *Em*-miR-71, and *Em*-miR-222-3p that inhibited nitrous oxide (NO) release by host macrophages[55], participated in immune process regulation of mouse macrophages[56], and modulated macrophage immune functions by regulating NO secretion through lipopolysaccharide (LPS)/TLR4 signaling pathway[54], respectively.

On the other hand, investigating the expression profiles of host circulating 84 microRNAs and their relevance to host immune cells functions revealed significant upregulation of eight miRNAs in patients with active cystic echinococcosis, compared to those with inactive cysts. Among them, miR-Let-7 and miR-26 previously showed direct roles in host immune response, e.g., macrophages proliferation and activation, and oxidative damage through targeting ILs 6, 10, and 13. Two miRNAs (miR-195-5p and miR-16-5p) were also implicated in promoting apoptosis in a variety of immune cells. Moreover, miR-30, and miR-223 exhibited essential role in regulating host innate immunity through type I INF signaling pathway[57].

Infection establishment: It was reported that *E. multilocularis* miRNAs disturbed the expression of four host genes (*ago1, ago4, tarbp2* and *xrn2*) essential for modulation of liver cell death and fibrosis[58]. Additionally, since *Em*-miR-71 was differentially expressed at several developmental stages with higher levels in protoscoles, a study demonstrated that Nemo-like kinase gene was its host target gene[59].

Diagnostic and prognostic biomarkers: To validate host miRNAs potential diagnostic biomarkers for hepatic alveolar echinococcosis (HAE), a Chinese study screened differentially expressed miRNAs, and quantitively determined their levels in liver tissue of infected patients. Compared to healthy control, there was significant increased expression of circulating host miR-483-3p. Its target was predicted as Lamin B receptor, which is a multi-transmembrane protein of nuclear membrane often used as a "reporter" of nuclear membrane dynamics. Therefore, it was suggested as a potential non-invasive biomarker for HAE for future studies[60].

Since recurrence is common after surgery and follow-ups become problematic, Iranian investigators quantitively determined two *E. granulosus* miRNAs (*Eg*-miR-71, and *Eg*-let-7) using real-time qPCR. The study was conducted on 30 hydatid cyst-infected patients before surgery as well as after 3 and 6 m. Results revealed significant down regulation of both miRNAs in both follow-ups, however; *Eg*-miR-71 had a higher expression level compared with *Eg*-let-7. The study claimed their study limitation, i.e., cross-reactivity with other helminths-derived miRNAs[61]. A study identified 7 *E. multilocularis* miRNAs in the sera of infected mice, among them only three (miR-10, miR-227, miR-71) were specifically amplified in all sera. The investigators proposed these miRNAs for future studies to validate their potentiality as diagnostic biomarkers for alveolar hydatid cyst[62]. In another study, levels of three miRNAs (miR-378a-3p, miR-101b-3p, and miR-192-5p) were significantly decreased 90 days post-infection (dpi) compared to 30 dpi in mouse livers[58]. In contrast, identification of *E. multilocularis* miRNAs in patient samples by real-time qPCR showed that they are not efficient diagnostic biomarkers for alveolar echinococcosis[63].

Regarding prognostic biomarker for follow-up after treatment, a study observed that hydatid cyst fluid was involved in the progression of fibrosis through regulation of host miR-19 expression that activated HSCs promoting hepatic fibrosis *via* increased TβRII levels, and extracellular matrix production. Accordingly, the investigators proposed host miR-19 an efficient treatment biomarker in intermediate hosts infected with *E. granulosus*[64].

Therapeutic roles: In *E. multilocularis*, a study analyzed the expression profile of miRNAs *in vitro*, and performed *in silico* functional analyses. Results revealed two highly expressed miRNAs in all developmental stages with essential roles in development, survival, and host-parasite interaction. They included miR-71, and miR-4989 that were absent in the host. The former was expressed in the germinal layer cells of metacestodes cultured *in vitro*, while miR-4989 was highly conserved in several cestodes. The investigators proposed them as potential drug targets that would help identify selective novel therapeutic drugs for treatment and control of alveolar hydatid cysts[65].

Cysticercosis

Based on several observations recorded for *T. solium* miRNAs and aided with the advanced exploration of its genome, a report summarized host-parasite interactions in neurocysticercosis. In naturally infected pig' brain, *C. cellulolosa* larva was surrounded by glial cells. The investigators demonstrated that while miR-124 regulated glial cells activation, miR-146 induced astrocyte-mediated inflammation through release of cytokines (IL-6 and TNF-α). The study also observed that miR-150 disturbed the blood brain barrier with increased vascular permeability. In addition, high expression of miR-155, miR-206, miR-223, and miR-511 were reportedly associated with progression of meningitis, and other neurological manifestations associated with chronic inflammations[66].

Later, to determine the role played by *Taenia* miRNAs in host immunomodulation during cysticercosis, a study constructed a small RNA (sRNA) library identified in *T. solium* and *T. crassiceps* genomes. The investigators observed that both *Taenia* spp. shared similar miRNAs, and miR-10-5p was the most abundant miRNA in both larval stages, followed by let-7-5p in *T. solium* and miR-4989-3p in *T. crassiceps*. However, among the miRNAs identified in adults, miR-001-3p was the most abundant in both species, followed by miR-002-3p in *T. solium*, and miR-003a-3p in *T. crassiceps*. Sequencing analyses and target prediction studies of *T. solium* larval miRNAs demonstrated that they have essential roles in host immunomodulation. Using RT-qPCR and ELISA assays of *T. solium* miR-10-5p and miR-let-7-5p, results revealed significant decreased expression of IL-12, IL-16, and TNF of cultured macrophages with moderate decrease of inducible NO synthase. The investigators suggested that both miRNAs down-regulated the production of pro-inflammatory cytokines, i.e., an immunosuppressive mechanism helping establishment of *T. solium* larva, and permanence inside their host[67].

Trichuriasis

Notably, *T. muris* is extensively used as a model to study host-parasite interaction in human trichuriasis. To better understand strategies employed by *Trichuris* to modulate host immune system, a study analyzed the sequenced *T. muris* released excretory/secretory (E/S) miRNAs using mass spectrometry. The investigators succeeded to identify 14 miRNAs that were compared with previously published miRNAs released in EVs of other nematodes. Results revealed high conservation at the functional level regarding host immune response, e.g., induction of Th2 immune response, suppression of proinflammatory cytokines, up-regulation of NO and IL-10, and suppression of $CD4^+$ proliferation[68]. One year later, another study analyzed EVs proteomic and genomic content and identified 56 miRNAs, among them 22 were not previously reported. The majority were involved in inflammation regulation and immunomodulation of host immune response. Additionally, the study demonstrated that miRNAs released in EVs were internalized by mouse colonic organoids suggesting their role in cell-cell communication within the intestinal epithelial cells[69].

Ascariasis

Using bioinformatics analysis, Solexa deep sequencing, and stem-loop RT-qPCR, Chinese investigators compared miRNAs identified in both genders of *A. suum*. The study identified 494 and 505 miRNAs in female and male adults, respectively, among them only ~30% (154 miRNAs) were detected

in both genders. Functional prediction of these miRNAs revealed involvement in survival, growth and development. While major sperm protein and nematode sperm cell motility protein were recorded predicted targets for male-specific miRNAs, ovarian message protein was for female-specific miRNAs[70]. One year later, similar results were obtained by another Chinese study when the investigators compared miRNA profiles of *A. lumbricoides* and *A. suum* females. Both species recorded high percentage of total reads (72.5%), while target and function prediction revealed a significant set of targets related to ovarian message protein, vitellogenin and chondroitin proteoglycan. The investigators concluded that both species represent a single species[71].

Later, American investigators conducted a study to quantitatively compare the expression of miRNAs from three regions of *A. suum* intestine. A total of 2,063 intestinal mRNA transcripts were identified targeted by miRNAs. Variant expression levels among the three intestinal regions were recorded, and the highest expression was observed from the anterior region. The investigators attributed their results to several issues: 1) nematodes intestine has to carry out essential functions related to nutrient acquisition, reproduction, and host-parasite interactions that influence host immune responses during infections; 2) the majority of encoding proteins are detected in the pseudocoelomic fluid that functions as a medium for transportation of yolk proteins utilized in the reproductive organs, and detoxification; and 3) E/S products are mainly released from the anterior intestinal region because it has a relatively high number of genes that are differentially expressed during infection[72].

Aiming to characterize EVs released by *A. suum* life stages and body parts, Hansen and her colleagues[73] conducted a study to evaluate the potential role of miRNAs cargo in host immunomodulation. Using transmission electron microscopy, the investigators demonstrated EVs release from the 3rd and 4th larvae and adults body fluids. Sequencing of EV-derived miRNAs identified transcripts that targeted host immune response including expression levels of several cytokines (IL-13, IL-25 and IL-33). A higher number of miRNAs was detected in L3; and 15 miRNAs were not identified either in L4 or in adults body fluid. This result was attributed to their essential role during larval migration with subsequent evasion of innate host immune response for the initial establishment and survival in their host. Among miRNAs involved in immunomodulation of host immune response were lin-4-5p, let-7-5p, miR-5350d-5p, miR-87a-3p and miR87b-3p[73]. Using *in silico* approach, Minkler and her colleagues[74] conducted an interesting study demonstrating that *Ascaris* EVs-derived circRNAs showed binding activity with both *A. sum* miRNAs as well as human host miRNAs. The investigators suggested that circRNAs can function as both endogenous and exogenous miRNA sponges to alter gene expression. In other words, circRNAs are enriched for miRNA binding sites to interact with host miRNAs influencing host gene transcription[74].

Toxocariasis

Using high-throughput RNA sequencing and bioinformatic analyses, a study identified 560 and 619 miRNAs in male and female *T. canis*, respectively. Among them, 218, and 277 miRNAs were transcribed exclusively in either gender, respectively. Functional prediction analysis of the gender-specific miRNAs revealed involvement in embryonic morphogenesis, and larval development. Besides, the investigators demonstrated involvement of miR-2305 and miR-6090 in reproduction, embryo development and larval development, whereas let-7-5p, miR-34 and miR-100 were involved in host-parasite interactions. Interestingly, the study also predicted involvement of miR-2861, miR-2881 and miR-5126 in drug resistance commonly reported in ascariasis[75].

Strongyloidiasis

In 2015, a German study hypothesized that the fate of *Strongyloides* life cycle stages whether in free living (Dauer) or parasitic status is determined by post-transcriptional regulatory mechanisms. Comparison between life cycles of three different nematodes *C. elegans* (free living), *Pristionchus pacificus* (necromenic) and *S. ratti* (parasitic) revealed the ability to form a Dauer stage representing an important step in the evolution toward parasitism. Sequencing miRNAs characterized in the three nematodes revealed a limited core set of 24 conserved miRNA families. Moreover, comparison between gene targets of miRNA expressed in Dauer and infective stages showed two miRNAs (miR-34, and miR-71) that serve as conserved post-transcriptional regulators of the Dauer/infective larvae fate. Accordingly, the investigators concluded that since the role of mRNA synthesis is not necessary for Dauer state, transcripts are accumulated during Dauer, and their activity is regulated by post-transcriptional regulators by miRNAs[76].

The first identification and characterization of miRNAs of *S. stercoralis* L1 and infective L3 in stool samples of infected patients was recently reported. The study observed 53%, 19%, 1% homology with those identified in *S. ratti*, *S. papillosus*, and *C. elegans*, respectively, while 44% were not previously reported. Using bioinformatics analysis, only few miRNAs were exclusively expressed in the infective L3 (miR-34a-3p, miR-34c-3p, miR-8397-3p, miR-7880M-5p, and miR-34B-3p). The investigators recommended further studies to validate their value in diagnosis[77].

Angiostrongyliasis

Since *A. cantonensis* can cause lethal eosinophilic meningoencephalitis in human, a Chinese study was conducted to identify potential non-invasive biomarker

for early diagnosis. Using Solexa deep sequencing and gene ontology (GO) classifications, the investigators identified 18 miRNAs with significant differential expression in L4 compared with L3. Among them, only 6 (miR-29a, miR-124, miR-125a, miR-146a, miR-101, and miR-185), were different from human- and mouse-derived miRNAs. The study determined their expression levels in serum of experimentally infected mice. Compared to normal mice, only miR-146a showed a significant higher expression level in infected mice. When assessed in 30 patients and 30 healthy controls, it exhibited 83% sensitivity and 86.7% specificity[78].

Trichinosis

Regulation of biological processes for survival and growth: In 2016, a Chinese study collected larvae from the skeletal muscle of naturally infected pigs to examine the miRNAs expression in trichinosis muscular phase. Using *C. elegans*, and *B. malayi* datasets, the investigators succeeded to identify one novel miRNA with 12 precursors detected in *B. malayi* genome, while no novel miRNA was identified using *C. elegans* dataset. The study recorded several conserved miRNAs including miR-1, let-7, miR-72, miR-87, and miR-124 that were involved in *T. spiralis* growth and metabolism[79]. In the same year, using comparative genomic approach, an Indian study identified 11 novel conserved miRNAs, and the investigators succeeded to predict their target mRNA genes. Results revealed that they were involved in various metabolic and enzymatic activity of *T. spiralis* biological processes (33%), and cellular components (29%), while the remaining (38%) were involved in several unidentified molecular functions[80].

Later, expression profile of miRNAs *T. spiralis* adults (intestinal phase), and larvae (muscular phase) were characterized. Those expressed in adults were identified in EVs, whereas miRNAs released in larvae were not encapsulated in skeletal muscle cells. Interestingly, one of those larval miRNAs was a homologue of mammalian miRNA-31 that shows essential roles in muscle development, i.e., it has an essential role in nurse cells formation. The investigators proposed it as a potential drug target for treatment of trichinosis muscular phase[81].

It is known that apoptosis is a vital protective mechanism played by pathogens to maintain host intestinal barrier function. To elucidate the mechanisms employed by *Ts*-miRNAs to interact with intestinal epithelial cells (IECs), a recent study selected miR-153 since one of its predicted gene targets is *bcl2* that regulate cell apoptosis. Using real-time qPCR and Western blotting, the study demonstrated that only *bcl2* was downregulated by *Ts*-miR-153 in IECs lines. Accordingly, it was concluded that *Ts*-miR-153 released in EVs of *T. spiralis* larvae induced cell apoptosis by targeting *bcl2* gene. Two mechanisms were postulated for *Ts*-miRNAs/IECs interaction, either reduction of mitochondrial membrane potential causing cell damage and substantial oxidative stress, or regulation of MAPK and tumor suppressing gene (*p53*) signaling pathways involved in cell apoptosis[82].

Immunomodulation of host immune response: A Chinese study investigated *T. spiralis* EVs immunoregulatory roles in experimental models with induced colitis using 2,4,6-trinitrobenzene sulfonic acid. The investigators observed that *Ts*-EVs significantly ameliorated induced colitis with reduction in intestinal epithelium barrier damage, and upregulation of the immunoregulatory cytokine expression in colon tissue. They also modulated the adaptive immune response through decreasing Th1 and Th17 cells, and increasing Th2 and Treg cells. The study also sequenced EV-derived miRNAs and demonstrated that several miRNAs were involved in immunomodulation of host immune response. Based on the study results, the investigators recommended future studies to further identify specific miRNAs released in *Ts*-EVs that might clarify their roles in host-parasite interactions during trichinosis[83]. Recently, A Chinese study investigated the role of *T. spiralis* EVs and their miRNAs cargo in immunomodulation of host immune response. The investigators demonstrated that *Ts*-miR-1-3p and *Ts*-let-7-5p, expressed in larvae-derived EVs activated the polarization of host macrophages to M2b type to evade the host's immune response through secretion of anti-inflammatory cytokines. Besides, the study demonstrated the essential role played by both miRNAs to inhibit the activation of host fibroblasts, and reduced collagen I that contribute to prevention of calcification of *Trichinella* capsule, i.e., long-term coexistence of the muscular phase[84].

Diagnostic biomarkers: Since circulating miRNAs are stably detectable components in blood and/or body fluids of infected hosts, Chinese investigators identified ten differentially expressed circulating mouse miRNAs. They equally included upregulated miRNAs (miR-467a-3p, miR-467d-3p, miR-376b-3p, miR-664-3p, and miR-292a-5p) and downregulated miRNAs (miR-199a-5p, miR-455-5p, miR-125b-5p, miR-125a-5p, and miR-615-3p) during different intervals post infection. The investigators recommended further studies to validate using upregulated miRNAs as potential promising biomarkers for trichinosis[85].

Diseases caused by filarial nematodes

Unlike free-living and intestinal nematodes, filaria encounter pronounced environmental changes when they transition between vertebrate and insect hosts. Therefore, they employed several molecular strategies to increase their ability to inhabit their hosts surviving and reproducing for long time. British reviewers claimed that filarial miRNAs are the major pathway for RNA interference, and they discussed methods utilized by several studies to identify, characterize, and predict functional roles of variant miRNAs. The reviewers

concluded the following issues: 1) the majority of filarial miRNAs were not conserved, i.e., they follow diverse evolutionary paths linked to various aspects of their system biology such as habitat, host and vector; 2) the majority of them showed stage- and gender-specific expression; 3) the most commonly identified and functionally characterized miRNA in adults and microfilaria was miR-71; 4) analysis of reported functional prediction showed that host genes involved in immune response were the target genes; and 5) additional factors influence regulation of secretion and plasticity of miRNAs cargo released in EVs. Two different routes were illustrated to describe proposed mechanisms of EVs release in *Brugia* and *Onchocerca* species. Regarding miRNAs diagnostic and prognostic applications, the review summarized several filarial miRNAs proposed potential biomarkers in diagnosis of lymphatic filariasis, onchocerciasis, and dirofilariasis. However, the reviewers discussed the challenges that limit their validation including technical methods and analysis, and results interpretation, i.e., studies used different cut-offs for defining a candidate miRNA[86]. Later, a recent review classified specific miRNAs identified in filarial nematodes into 1) gender-biased miRNAs expression to regulate survival, growth, and differentiation; and 2) miRNAs cargo in EVs to alter host immune response during infection[11].

Lymphatic filariasis

Stage- and gender-specific miRNAs: It was reported that miRNAs 92, 153, 2a, 5366, and b5842 were highly expressed in microfilaria, whereas they differ in adults. In females, those with higher expression included miRNA-5866, and miRNA-9536 to regulate expression of genes encoding nuclear receptors, serpin activity, and structural cuticle molecules required for embryogenesis, larval development, and cuticle molting or synthesis, respectively. In males, miRNAs 283, 2e, 57, and 5838 were abundant regulating genes encoding protein kinases, phosphatases, and sperm proteins[87]. The specific *B. malayi* miRNAs (miRNA-5364) was essentially involved in several biological processes such as sexual differentiation, apoptosis, and host invasion. Besides, it played a specific role in L3 transmission from mosquito to mammalian host[88].

Survival, growth, and differentiation: The conserved miRNA in helminths (miRNA-71) is involved in longevity, stress resistance, and neuron development. Therefore, it was hypothesized that it modulated genes expression involved in protein kinases for insulin-like pathway, antioxidants, and metabolic enzymes necessary to extend adults and microfilaria lifespan[89].

Infection establishment: Since *Bm*-miRNA-71 was released by EVs, American investigators observed its internalization by host immune cells to induce NO production modulating host miRNAs linked to inflammation. In mice experimental model, *Bm*-miR-71 induced overexpression of host miR-125b-5p, miR-146a-5p, and miR-378-3p in macrophages with increased inflammatory response through IL-4 production. The study suggested that IL-4 production facilitated the activation of suppressive macrophages leading to immunosuppression observed in some cases of lymphatic filariasis with increased inhibition of other cellular processes during infection[90]. Recently, several *B. malayi* miRNAs were reportedly released in EVs immunomodulated host responses; among them were *Bm*-miRNA-100b, and *Bm*-miRNA-34. The investigators suggested that both miRNAs downregulated molecules were involved in the mTOR pathway, an essential signaling pathway for immune regulation and cell proliferation and differentiation[91].

Diagnostic biomarkers: Due to their high expression in *B. pahangi*-infected dog sera, a study proposed host circulating miR-71 and miR-34 as potential diagnostic biomarkers[92].

Loiasis, onchocerciasis, and dirofilariasis

All studies conducted in *L. loa*, *O. volvulus*, and *D. immitis* miRNAs dealt with their use as potential diagnostic biomarkers. Utilizing bioinformatics analysis, the investigators identified 22 miRNAs in plasma from *L. loa*-infected baboons. None was previously reported in a study conducted by the same group of investigators[92] when identified in circulating blood of *D. immitis*-infected dogs and *O. volvulus* infected patients. Deep-sequencing of the identified miRNAs revealed that 8 miRNAs were identical in at least two infections, and 9 miRNAs had low homology in the investigated filarial nematodes. Accordingly, the investigators concluded that filarial miRNAs circulating in their hosts are specific, and further studies were recommended to validate their use as potential diagnostic biomarkers[93].

By sequencing of host circulating miRNAs and bioinformatics analysis, 21 *O. volvulus* miRNAs were identified in the sera of infected patients. The study recommended future studies to validate use of host circulating miRNAs in diagnosis of onchocerciasis[92]. Sequencing analysis of the total RNA extracted from bovine onchocercomata nodule fluid, and blood samples collected from *O. volvulus*-infected patients revealed 62 and six miRNAs, respectively. The six miRNAs (members of miR-71, bantam family and miR-100 family miRNAs) identified circulating in human sera had orthologs in other filarial nematodes, i.e., they are conserved miRNAs. Among them, four miRNAs were previously reported in blood samples collected from mice infected with *Litomosoides sigmodontis*. The investigators recommended further studies investigating miRNAs as new non-invasive potential biomarker for diagnosis of onchocerciasis[94].

In contrast, two studies denied efficient use of miRNAs for diagnosis in onchocerciasis. The first study investigated whether circulating miRNAs are

specifically expressed in measurable quantities. Serum samples were collected from 23 individuals with query nodules for differential diagnosis, and 20 patients with onchocercomata nodules. After miRNAs extraction, the investigators evaluated a set of 17 miRNAs of variant filarial nematodes using RT-qPCR. Results revealed that only seven miRNAs (two *B. malayi*, five *O. volvulus* miRNAs) were quantitatively detected. These included *Bm*-miR-236-1, *Bm*-miR-71, *Ov*-miR71-22nt, *Ov*-miR-71-23nt, *Ov*-miR-100d, *Ov*-bantam-a, and *Ov*-miR-87-3p. Melting curve analysis showed that signals recorded for *B. malayi* miRNAs, and *O. volvulus* miR-87-3p, and miR-71 variants were non-specific. The remaining two miRNAs only showed positive signal in one or few samples with values below the cutoff. Accordingly, the study concluded that measurement of miRNAs was not an efficient assay to conform treatment success in onchocerciasis[95]. Later, a recent study screened circulating *O. volvulus* miRNA in serum samples collected from infected patients 4-, 12- and 21-months after microfilaricidal treatment. The investigators conducted their study on 18 patients administered ivermectin (4), doxycycline (9), and combined treatment (5) using circulating filarial miRNAs (*Bm*-lin-4, *Ov*-miR-lin-4, and *Ov*-miR-71-5p). The study also investigated additional biomarker, *Ov*-150 repeats, since it was previously assigned with a high-quality *O. volvulus* genome assembly, and highly abundant DNA repeat families identified in *O. volvulus* genome. Using RT- qPCR, results revealed detection of the investigated circulating biomarkers in only two of 72, and 47 samples in patients without and with microfilaridermia, respectively. However, *Ov*-150 DNA repeats was detected in eight baseline samples, and the positives samples declined post treatment. Since miRNAs were variably detectable at low to undetectable concentrations in host plasma, the investigators concluded that circulating miRNAs were insufficient as diagnostic or prognostic biomarkers for evaluation of treatment efficacy in onchocerciasis[96].

Bioinformatics analysis of host circulating miRNAs revealed several *D. immitis* miRNAs (~200) with high expression in infected dog plasma. However, their expressions did not significantly correlate with microfilaria count. The study proposed them potential diagnostic biomarkers for further validation[92].

CONCLUDING REMARKS

1. Since miRNAs are one of the RNA interference pathways, they fine-tune several cellular biological processes through regulation of cellular transcription. Therefore, they orchestrate gene expression to keep specific rhythm and dynamics during cell development, i.e., switch pacemakers that turn on or off to specify cellular transcription at a specific time. During the last two decades, identification and characterization of parasite-specific miRNAs and their target genes became a successful approach to identify diagnostic and prognostic biomarkers, as well as novel therapeutic and protective agents to control neglected tropical diseases.
2. Their release in EVs is essentially contributed in cell-to-cell communication, and host-parasite interactions through their influence on host miRNAs regulating host immune cells. Besides, development of diagnostic and prognostic biomarkers detected in blood and body fluids are ideal diagnostic and/or prognostic non-invasive procedures. Infection probability, and disease pathogenicity are also regulated by miRNAs cargo released in EVs through mediation of post-transcriptional regulation of genes involved in disease pathogenesis, and modulation of host miRNAs involved in controlling immune response against parasite replication and proliferation.
3. The most important observation is their differential expression among parasite's developmental stages and between both genders. This confirms the specific roles played by miRNAs in regulation of gene expression, i.e., each miRNA has a certain function in an individual stage at a specific time.
4. Host immunomodulation to establish disease pathogenesis is the most important role described in almost all parasites. It was observed that miRNAs released into EVs were internalized by host macrophages with subsequent modulation of host signaling pathways in the favor of parasite replication and infection establishment. Several mechanisms were proposed; decreased T cell differentiation into Th2 subpopulations, production of pro-inflammatory cytokines, downregulation of NF-κB pathway, inhibition of TLRs-mediated inflammation, and regulation of both INF-I signaling pathway and NO production.
5. Hepatic fibrosis reported in schistosomiasis and clonorchiasis is a good example to describe the role of parasite-derived miRNAs in disease pathogenesis. Accordingly, *Schistosoma* miRNAs were either those with pro-fibrogenic or anti-fibrogenic roles. Pro-fibrogenic miRNAs included those that enhance HSCs activation, and activate SMAD signaling pathway. Anti-fibrogenic miRNAs included those that inhibit HSCs activation or induce their apoptosis, target TβR1, and regulate macrophages transformation from M1 to M2/IFN-γ signaling pathway. In addition, use of combined host miRNAs proved a potential biomarker for grading hepatic fibrosis.
6. Several parasite-derived miRNAs were investigated and reportedly succeeded in predicting cancer bladder, hepatocellular carcinoma, and cholangiocarcinoma in patients infected with *S. haematobium*, *S. japonicum*, and *C. sinensis*, respectively.
7. The miRNAs proposed as potentially therapeutic were described in amelioration of hepatic fibrosis associated with schistosomiasis *japonicum* (*Sj*-miRNAs), treatment and control of alveolar

hydatid cysts (*Em*-miRNA), and induced colitis in experimental models (*Ts*-miRNA). Additionally, several studies utilized the approach of cross-species regulation of genes suppressing cancer to validate the potential anti-cancer activity of *S. japonicum* miRNAs.

8. Being non-invasive biomarkers, several parasite-derived and host miRNAs were investigated for their diagnostic and/or prognostic potentiality in hepatic alveolar echinococcosis, strongyloidiasis, eosinophilic meningoencephalitis, muscular trichinosis, and diseases caused by filaria. However, there is much debate due to several limitation including cross-reactivity with other helminths-derived miRNAs, detection variability in host plasma, and much controversy regarding the cut-off value.

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