# Host–*Plasmodium* interaction: role of RNAi

**Rohit Shrivastava[1,2] and S. Rajasubramaniam[1,*]**

[1]ICMR-National Institute of Research in Tribal Health, Jabalpur 482 003, India
[2]Present address: Department of Life Science, Ben-Gurion University of the Negev Beer-Sheva, Israel

**Malaria is a global health problem that afflicts an estimated 90 million people worldwide. Significant improvement in the understanding of *Plasmodium* life cycle has been achieved, yet multitude of clinical effects seen in malaria remains unanswered. MicroRNAs (miRNAs) have been implicated in the pathogenesis of malaria. These miRNAs act as post-transcriptional regulators and control host's cellular factors needed for *Plasmodium* multiplication and suppress immune responses. Dysregulated miRNA expression has been linked to malaria pathogenesis through modulation of signalling pathways involved in processes such as proliferation, metabolism, gene expression and immune response in the host. In humans, *Plasmodium* infection severely affects hepatic functions and erythrocytic life span while severe infection with *P. falciparum* can lead to cerebral pathology. The present review aims to gain insight into the contribution of miRNAs to the exo-erythrocytic and erythrocytic stage pathology of *Plasmodium* infection and coordinated regulation in *Plasmodium*-mediated progression to cerebral pathology.**

**Keywords:** Cerebral pathology, MicroRNAs, *Plasmodium*.

MALARIA is caused by a member of one of the most virulent *Apicomplexan* parasite of the *Plasmodium* genus[1]. *Plasmodium* is a genus of protista parasite among which *P. falciparum*, *P. vivax*, *P. ovale* and *P. knowlesi* cause human malaria[2]. *Plasmodium* is an obligatory parasite that replicates inside parasitophorous vacuole and exhibits a complex lifecycle alternating between two hosts, namely an insect vector (primary/definitive host), where sexual reproduction occurs and a vertebrate host (secondary host) the site of asexual reproduction (Figure 1)[2]. *Plasmodium* life cycle has two phases in the secondary host, namely exo-erythrocytic phase and erythrocytic phase. The exo-erythrocytic or the hepatic phase is clinically an asymptomatic stage wherein sporozoite invades hepatocytes, utilizes host's hepatic proteins and lipids for growth and undergoes multiple rounds of replication to finally produce merosomes containing multiple merozoites (Figure 1)[3,4]. Onset of blood stage infection is marked by the release of merozoites from merosomes in pulmonary capillaries of lungs[5].

*For correspondence. (e-mail: raja.rmrct@gmail.com)

Malaria is endemic to sub-Saharan African region and Southeast Asia and remains a major public health issue globally[6]. An estimated 89–90 million people worldwide are afflicted with malaria[6]. In India, malaria accounts for about 1 million cases annually with half of them due to *Plasmodium falciparum* (*Pf*) infection[6,7]. Majority of malaria-induced deaths are due to *Pf* infection that leads to a major complication known as cerebral malaria[8]. *Pf* infection is a major risk factor for cerebral malaria related encephalopathy and respiratory distress[8]. Although infected host cells have been immensely studied, little is known about *Plasmodium*-mediated molecular alterations occurring in the host cell. The present review is based on evidences from multiple studies investigating microRNA deregulation during stages of malarial infection and attempts to identify plausible mechanism of various *Plasmodium*-mediated manipulation of host molecular machinery.

## RNA interference and microRNAs

MicroRNAs (miRNAs) are small evolutionary-conserved non-coding RNAs that regulate gene expression through RNA interference (RNAi)[9]. RNAi is an evolutionary-conserved process well evident in eukaryotes that post-transcriptionally regulate gene expression through RNA binding proteins (RBPs)[10]. RNAi pathway can be triggered by double stranded viral RNA, foreign DNA or miRNAs. Viral integrated shDNA inserts are transcribed into long highly structured single strand RNAs (ssRNAs). These ssRNAs are cleaved by Drosha into smaller precursor shRNAs. Precursor shRNA molecules are transported to cytoplasm for maturation into siRNA by Dicer protein. Mature siRNA is loaded into RNA induced silencing complex (RISC) (Table 1). Of the two strands of siRNA, one strand is degraded termed as passenger strand and the other guide strand hybridizes with the target mRNA[11,12]. Perfect match between the siRNA and mRNA target results in proteolytic cleavage of the target mRNA via argonaute protein leading to gene silencing Figure 2 *a* (ref. 12). An imperfect match leads to translation inhibition. Biogenesis of miRNA is similar to shRNA; maturation of miRNA requires RNase II enzymes Drosha and Dicer. Guide strand of the mature miRNAs are then incorporated into effector RISC complex and hybridizes with 3′ untranslated region (UTR) of target mRNA and causes either degradation of mRNA by destabilizing mRNA

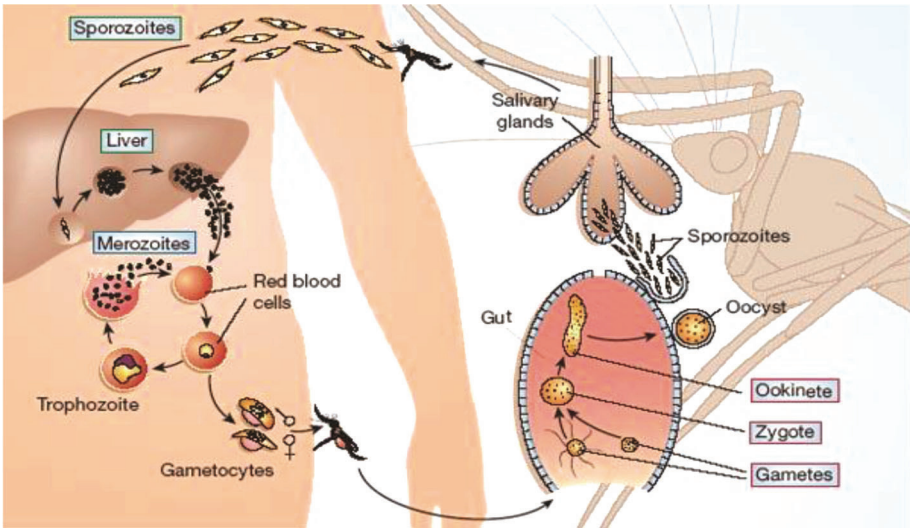


**Figure 1.** *Plasmodium* life cycle. Three different stages of *Plasmodium* life cycle, pre-erythrocytic or hepatic phase, erythrocytic phase and mosquito phase, are shown. Image adapted from ref. 78.

**Table 1.** Function of RNAi components. Functional role of proteins involved in biogenesis and transport of miRNAs and formation of RISC that is required for miRNA mediated gene regulation

| Gene | Function |
|---|---|
| *Xpo-5* | Ran-GTPase based transporter of pre-miRNA from nucleus to cytoplasm[79]. |
| *Ago-2* | It forms the catalytic portion of RNA induced silencing complex (RISC)[79] |
| *Dicer* | An endoribonuclease required for processing of pre-miRNA into mature miRNA and facilitates RISC formation[79] |
| *TRBP* | It is a human immunodeficiency virus transactivating response RNA-binding protein, regulates Dicer[79] |
| *Drosha* | A class 2 RNase III enzyme required for processing long pri-miRNA into pre-miRNA[79] |

transcript or suppresses translation by sequestration into processing bodies (P-bodies) that have a fundamental role in mRNA decay Figure 2 *b*[10,13]. Contrary to the canonical function, upon subjecting cells to stress, miRNA interact with the 5′ UTR of mRNA and promote translation[14,15]. M*iR-369-3* (MIMAT0000721) has been shown to recruit specific ribonucleoproteins such as fragile X mental retardation-related protein 1 and Argonaute 2 to AU rich element of tumour necrosis factor $\alpha$ and activates translation contrary to their primary role of repression[16,17]. Some miRNAs were observed to carry distinct hexanucleotide terminal motifs, like *miR-29b*, which directs their nuclear localization[18]. Nuclear enrichment of these miRNAs enables them to affect transcription at promoter level through direct interaction with complimentary DNA sequences to form triple helix causing gene silencing. These evidences suggest that different miRNAs function at different sub-cellular compartments[19]. Moreover, *miR-373* has been shown to bind to *CDH1* promoter and stimulate transcription[16]. Further, miRNAs regulate epigenetic machinery by targeting components of enzymatic complexes involved in epigenetic regulations[20]. These evidences suggest that miRNAs although small, can regulate complex functions.

Recently, microRNAs (miRNAs) have emerged to be critically implicated in the etiology of numerous diseases such as HCV infection, HIV, cancer, etc. Meanwhile, studies have recognized the intricate involvement of miRNAs and RNAi machinery in the dynamic parasite–host interaction. List of miRNAs discovered is growing steadily and until now there are 2578 known miRNAs in humans[21] and more than 1900 miRNAs in mouse[22]. Each of these miRNA is predicted to target hundreds of transcripts of protein-coding genes and simultaneously a single mRNA could be regulated by several miRNAs. Recent evidences indicate the critical role of miRNAs in immune response regulation[23]. Numerous miRNAs have been identified to be involved in the regulation of innate and adaptive responses including inflammation, development, differentiation, activation of B and T cells and antibody switching[24,25]. Chen *et. al.*[26] demonstrated that miRNAs act as regulators of gene expression following parasitic infections and established their role in immune response. They demonstrated that *Cryptosporidium parvum* infection regulates let-7-mediated toll-like receptor 4 (TLR4) signalling in cholangiocyte immune response[26]. Further, they showed that following *C. parvum* infection expression of let-7 and miR-98 are down-regulated that in

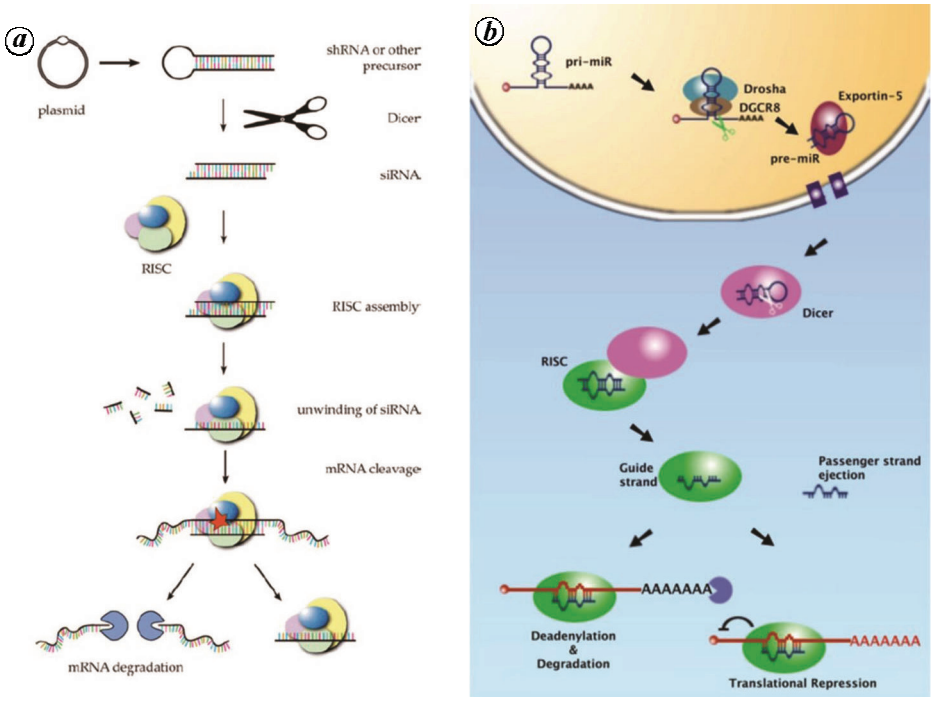


**Figure 2.** RNAi machinery. Mechanism of action of siRNA (***a***) and of miRNAs (***b***). Image adapted from refs 11 and 12.

turn lowers the expression of suppressor of cytokine signalling (SOCS) protein and reduced SOCS1 protein leads to lower IFN-$\gamma$ regulation and T-cell differentiation[27,28]. In addition, *Toxoplasma gondii* has been shown to inhibit host cell apoptosis by STAT3-mediated upregulation of anti-apoptosis miRNAs[29]. Furthermore, miRNAs are also known to regulate growth, stress response, metabolism, infection and gene expression in both liver[30–32] and brain[33–36] – two organs that are critically involved in the pathogenesis of malaria.

## MicroRNAs in malaria

Several studies aimed at understanding the influence of malaria parasites on host's miRNA expression profile have revealed disruption of cellular homeostasis due to infection. This is challenged by the change in cellular miRNA expression to inactivate the parasite proteins or host factors critical for parasite survival[37,38]. Accumulating evidences point to exploitation of the host RNAi machinery by the pathogens to manipulate miRNA expression favouring their growth and survival as seen in *Toxoplasma* and *Cryptosporidium*, members of *Apicomplexan* phylum[37]. Moreover, it is important to note that *Cryptosporidium* lacks a functional RNAi machinery, yet, successfully manipulates host miRNA expression enabling its survival[37,39]. Interestingly, few viruses are also known to encode viral microRNAs that lack functional components of RNAi machinery. It was found that murine $\gamma$-herpes virus MHV68 synthesizes viral precursor-miRNAs (pre-miRNAs) independent of *Drosha* by using tRNase 3 endonuclease; tRNaseZ, the viral pre-miRNAs are then processed by host Dicer in the cytoplasm[40,41]. Further, *Plasmodium* genome encodes a number of putative proteins that contain domains similar to that found in human RNase, indicating the existence of similar mechanism in *Plasmodium*. Furthermore, evidence of miRNA biogenesis from cytoplasmic RNA viruses suggests that a low fraction of miRNAs may be processed by the host RNAi machinery available to *Plasmodium* during host cell mitosis during nuclear membrane cleavage[42]. However, we were unable to find any experimental evidence to support this observation. It has been observed in a number of studies that *Pf* infection leads to enhanced activation of mitogen-activated protein kinase/extracellular signal-regulated kinases (MAPK/ERK) involved in inflammatory response[43]. It was observed by Paroo *et al.*[44] that TRBP is phosphorylated by MAPK/ERK pathway and phosphorylation of TRBP regulates the recruitment of Dicer complex to Ago-2 that enhances miRNA maturation[44]. Ago-2 is also regulated via phosphorylation through MAPK[45]. Furthermore, expression of transforming growth factor-beta (TGF-$\beta$) in malaria regulates the pro-inflammatory cytokines[46]. TGF-$\beta$ is also involved in the recruitment of SMAD proteins that interacts with RNA helicase p68 (DDX5) in Drosha complex suggesting its role in miRNA biogenesis[47].

In general the parasite transcripts remain protected from degradation by RNAi machinery in parasitophorus vacuole. Further, with the presence of internal ribosome

entry site (IRES) the canonical mode of action of miRNAs, i.e. decapping, becomes redundant. So it is not clear why *Plasmodium* needs or choose to manipulate host RNAi machinery. The only plausible explanation comes from identification of non-canonical mechanisms adopted by miRNAs in the absence of RNAi machinery (discussed later). The ability of miRNA to integrate itself into parasite transcripts through mechanisms unknown is suggestive of co-evolution of the host and *Plasmodium*.

*Plasmodium* downregulates certain miRNAs with the potential of inhibiting parasitic translation, down-regulates miRNAs that target host cell proliferation, metabolism and survival, while it upregulates miRNAs that target proteins involved in immune response. These findings indicate a high probability that by orderly manipulation of both MAPK/ERK and TGF-$\beta$ pathways, *Plasmodium* may manipulate expression of miRNAs to favour its growth and survival.

## MicroRNAs in exo-erythrocytic stage of malaria – hepatic pathology

Clinically asymptomatic hepatic stage of malaria is critical to both the parasite as well as host and provides ample targets for the development of therapeutic molecules and vaccine. Although hepatic stage is the most studied stage and crucial, the dynamic interactions between host hepatocytes and parasite remain poorly understood. Recently, Delić *et al.*[48] provided the experimental evidence of murine hepatic reprogramming of miRNA expression by *Plasmodium chabaudi*. They observed that mice were able to survive primary infection with *P chabaudi* and when re-challenged, 80% mice self-healed and survived[48]. They found that protective immunity in mice against *P. chabaudi* was associated with up-regulation of 3 miRNAs (miR-26b, MCMV-miR-M23-1-5p and miR-1274a) and down-regulation of 16 miRNAs (miR-101b, let-7a, let-7g, miR-193a-3p, miR-192, miR-142-5p, miR-465d, miR-677, miR-98, miR-694, miR-374*, miR-450b-5p, miR-464, miR-377, miR-20a* and miR-466d-3p) (Table 2)[48]. Among these downregulated miRNAs, miR-142-5p is known to target T cell activation and differentiation thus inducing immune response[49,50]. Furthermore, downregulated miR-192-3p regulates tumour specific hypermethylation[51] and miR-101b regulates *de novo* DNA methylation by targeting DNA methyltransferase 3A (DNMT3A)[52]. The self-healing observed in re-infected mice was associated with increased levels of circulating IgG2a and IgG2b antibodies and thus evidence to induction of protective immunity[48,53]. Therefore, *P. chabaudi* induced sustained alteration of hepatic miRNA profile coincides with gain of protective immunity that may be due to epigenetic regulation of gene expression.

Hammerschmidt-Kamper[54] investigated influence of *P. berghei* (*Pb*) NK65 on host hepatic RNAi machinery using wild type (WT), radiation attenuated (RAS) and genetically attenuated parasite (GAP) at 24 and 40 h post infection. Influence of *Pb*NK65 WT, RAS and GAP strains on RNAi machinery was evaluated by transcription and protein expression analysis of RNAi component (exportin-5, argonaute, Dicer and Drosha). Table 3 clearly indicates significant down-regulation of RNAi components induced by attenuated parasite strain.

MiRNA expression profiling of PbNK65 WT, GAP and RAS infected livers at 24 and 40 h post-infection revealed 33 dysregulated miRNAs (20 miRNAs upregulated and 13 miRNAs down regulated; Table 2)[54]. It is interesting to note that the number of miRNAs down regulated were relatively low compared to upregulated ones. In fact, only miR-21 has been consistently upregulated in all experimental conditions (Figures 3 and 4)[54]. Bioinformatic analysis of dysregulated miRNAs revealed pathways affected which included apoptosis, DNA damage, immune response, cell adhesion, lipid metabolism and signal transduction[54]. In general, miR-21 is classified as 'OncomiR' as its dysregulation leads to cancer[55]. Thus, identification of a similar functional correlation to miR-21 will be of critical importance to the infection process in malaria.

Upregulated miR-21 and miR-25 in WT 24 h post infection are known to regulate innate immune response[50]. Moreover, miR-21 regulates apoptosis[56], antigen activated CD8+ T cell population and regulates TLR signalling involved in innate immune response[57], whereas miR-25 regulates TNF-$\alpha$ indicating a role in inflammatory responses[58].

However, it is intriguing to know why cells promote a varied change in miRNA signature that makes it susceptible to pathologic outcomes. Study revealed that infection with attenuated parasite leads to significant down-regulation of RNAi machinery components, exportin-5 (Xpo-5), argonaute (Ago), Dicer, Drosha and TRBP and concomitant protein levels. Although studies investigating parasite influences on RNAi machinery are scarce, studies evaluating viral influences on RNAi machinery have clearly demonstrated reduced transcription and translation of Drosha in cells infected with Hepatitis B virus[59] and low Dicer transcription in hepatocellular carcinoma[60]. These results incriminate sensitivity of RNAi machinery to cellular invasion by parasites. But the fundamental question that remains to be answered is why WT parasitic infections fail to down-regulate RNAi machinery that is observed in attenuated *Plasmodium* strains. The only plausible explanation that emanates is that parasite and host during evolution may have co-adapted to favour their joint survival, thus suggesting that *Plasmodium* infection may be multi-factorial interaction involving hepatocytes and parasite.

**Table 2.** List of miRNAs involved in response to malarial infection

| microRNA | Accession no | Sequence | Function | Reference |
|---|---|---|---|---|
| miR-369-3p | MIMAT0000721 | AAUAAUACAUGGUUGAUCUUU | Regulates TNF$\alpha$ | 17 |
| miR-98 | MI0000100 | UGAGGUAGUAAGUUGUAUUGUU | Regulates SOCS1, IFN$\gamma$ and T-cell differentiation | 27, 28 |
| miR-26b | MI00000575 | UUCAAGUAAUUCAGGAUAGGU | Regulates TNFα/NF-$\kappa$B signalling and IL-6 expression | 80 |
| MCMV-miR-M23-1-5p | MIMAT0005542 | CUCGGUACGGACGGGGAACCGU | Regulates dendritic cell activation | 81 |
| miR-1274a | | Dead miRNA | Unknown function | |
| miR-101b | MI0000649 | UCGGUUAUCAUGGUACCGAUGCU | DNA methylation | 52 |
| let-7a | MI0000060 | UGAGGUAGUAGGUUGUAUAGUU | Regulates TLR-4, IL-6 and 10 | 82, 83 |
| let-7g | MI0000433 | UGAGGUAGUAGUUUGUACAGUU | TNF$\alpha$ induction | 84 |
| miR-193a-3p | MIMAT0000459 | AACUGGCCUACAAAGUCCCAGU | Regulates NF-$\kappa$B pathways and inflammation | 85 |
| miR-192-3p | MIMAT0017012 | CUGCCAAUUCCAUAGGUCACAG | Tumour specific hypermethylation | 51 |
| miR-142-5p | MIMAT0000154 | CAUAAAGUAGAAAGCACUACU | Regulates IL-6, IL6ST, TLR2, PGE2 and TNF | 86 |
| miR-465d | MIMAT0029880 | UAUUUAGAAUGGUACUGAUGUGA | LPS regulated innate immune response | 87 |
| miR-677 | MIMAT0003451 | UUCAGUGAUGAUUAGCUUCUGA | Regulates lymphocyte maturation at common lymphoid progenitor stage | 88 |
| miR-694 | MIMAT0003474 | CUGAAAAUGUUGCCUGAAG | Regulates Map1s and IL-6 and inflammatory response | 89, 90 |
| miR-374* | MIMAT0003728 | GGUUGUAUUAUCAUUGUCCGAG | Regulates regulatory T cells by IL-10 | 91 |
| miR-450b-5p | MIMAT0003511 | UUUUGCAGUAUGUUCCUGAAUA | Regulates IL-27 and JAK/STAT pathway in dendritic cells | 92 |
| miR-464 | | Dead miRNA | Unknown function | |
| miR-377 | MIMAT0000741 | AUCACACAAAGGCAACUUUUGU | Regulates NF-$\kappa$B pathways by E2F3 and MAP3K7 | 93 |
| miR-20a* | MIMAT0004627 | ACUGCAUUACGAGCACUUAAAG | Regulates TCR signalling and cytokine production | 94 |
| miR-466d-3p | MIMAT0004931 | UAUACAUACACGCACACAUAG | Regulates Nfat5 signalling | 95 |
| miR-21 | MIMAT0000530 | UAGCUUAUCAGACUGAUGUUGA | Innate immune response | 56, 57 |
| miR-25 | MIMAT0000652 | CAUUGCACUUGUCUCGGUCUGA | Innate immune response | 56, 57 |
| let-7i | MIMAT0000122 | UGAGGUAGUAGUUUGUGCUGUU | Innate immune response, TLR4 | 56, 57 |
| miR-27a | MIMAT0000537 | UUCACAGUGGCUAAGUUCCGC | Inflammatory response | 58,59 |
| miR-150 | MIMAT0000160 | UCUCCCAACCCUUGUACCAGUG | Regulates Myc-b, Lymphocyte development, immune response | 56, 57, 63 |
| miR-126 | MIMAT0000137 | CAUUAUUACUUUUGGUACGCG | Regulates vascular cell adhesion molecule 1 | 63 |
| miR-210 | MIMAT0000658 | CUGUGCGUGUGACAGCGGCUGA | Regulates HIFα | 63 |
| miR-155 | MIMAT0000165 | UUAAUGCUAAUUGUGAUAGGGGU | Regulates blood brain barrier integrity | 96 |
| miR-451 | MIMAT0001631 | AAACCGUUACCAUUACUGAGUU | Inhibition of parasite growth | 71 |
| miR-223 | MIMAT0004570 | CGUGUAUUUGACAAGCUGAGUU | Inhibition of parasite growth | 71 |

**Table 3.** Influence of PbNK65 WT, RAS and GAP strains on RNAi components. Transcription expression analysis of RNAi component (Xpo-5, Ago-2, Dicer, TRBP and Drosha) was expressed as fold change based on the study of Hammershmidt-Kamper[55]. The changes are represented by black arrow-downregulated (↓) while protein expression of only Xpo-5 and Drosha was available, expressed as percentage and represented by red arrow-downregulated (↓). Parallel arrow (→) represents slight change and double arrows (↓↓) represent strong change

| Gene | WT | GAP | RAS | Time post infection (h) |
|---|---|---|---|---|
| Xpo-5 | ↓30–70% | → | ↓30–70% | 24 |
| | ↓ 30–70% | ↓ 8 fold, → | ↓8 fold, ↓↓ | 40 |
| Ago-2 | | | | 24 |
| | | | → | 40 |
| Dicer | | | | 24 |
| | ↓2–3 fold | ↓2–3 fold | ↓2–3 fold | 40 |
| TRBP | | | | 24 |
| | | ↓2–3 fold | ↓2–3 fold | 40 |
| Drosha | ↓51% | ↓2 fold | ↓2 fold, ↓64% | 24 |
| | ↓3–4 fold, ↓20–25% | ↓3–4 fold, ↓20–25% | ↓ 3–4 fold, ↓20–25% | 40 |

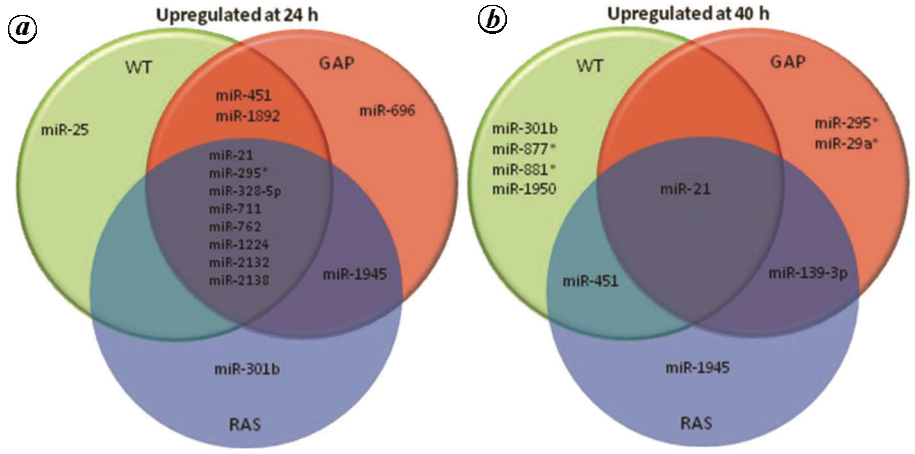


**Figure 3.** Differential and overlapping expression of upregulated hepatic miRNAs between PbNK65 WT, GAP and RAS at both 24 h and 40 h post infection. ***a***, Hepatic miRNA expression profile 24 h post infection with PbNK65 WT, GAP and RAS. ***b***, Hepatic miRNA expression profile 40 h post-infection with PbNK65 WT, GAP and RAS. Data taken from ref. 54.

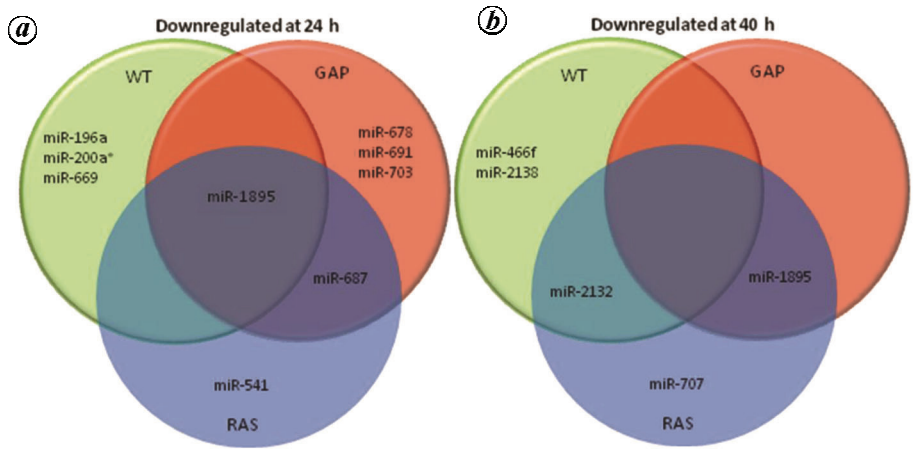


**Figure 4.** Differential and overlapping expression of downregulated hepatic miRNAs between PbNK65 WT, GAP and RAS at both 24 h and 40 h post infection. ***a***, Hepatic miRNA expression profile 24 h post infection with PbNK65 WT, GAP and RAS. ***b***, Hepatic miRNA expression profile 40 h post infection with PbNK65 WT, GAP and RAS. Data taken from ref. 54.

## MiRNAs in cerebral pathology

Cerebral pathology in malaria is a complex and multifactorial phenomenon which involves apoptosis, immune modulation and inflammation[61], cytoadherence[62] and hypoxia. Role of miRNAs in cerebral pathology was tested using murine experimental cerebral malaria (ECM) model. It was observed that six miRNAs (let-7i, miR-27a, miR-150, miR-126, miR-210 and miR-155) showed dysregulation of which only let-7i, miR-27a and miR-150 showed significantly altered expression[63] (Tables 2 and 4). Members of let-7 family are known to modulate innate immune response[26]. In ECM model, let-7i was upregulated that has earlier been shown to induce immune response in cholangiocytes by regulation of toll-like receptor 4 (TLR4) translation[26]. MicroRNA Let-7i (MI0000434) is also important for LPS induced dendritic cell maturation by regulation of SOCS1 (Tables 2 and 4)[64]. SOCS1 reduces LPS-mediated IL-6 production by regulating JAK/STAT pathway[65]. In T cells, let-7i upregulation correlated with increased IFN-$\gamma$ expression through inhibition of TLR4 and induced Th1-mediated immune response[66]. MiR-27a is known to regulate diverse cellular functions such as apoptosis, mitochondrial membrane potential and sensitivity to TNF$\alpha$. It also regulates the expression of fas associated death domain (FADD) required for T cell proliferation and NF-$\kappa$B signalling, thus regulating inflammatory response[67]. Overexpression of miR-27a causes TNF-$\alpha$ mediated apoptosis through extrinsic death pathway[67]. Moreover, upregulation of miR-27a in CD8+ T cells suggests their role in T cell development[68] (Tables 2 and 4). Finally, miR-150 is known to regulate proliferation, development and differentiation of B and T cells by targeting transcription

**Table 4.** Showing predicted genes targets of respective miRNAs. Since every software uses different parameters for prediction, results from softwares varied considerably; so only the predicted targets with highest scores are presented in the table. We used four different miRNA target prediction softwares based on different algorithms, namely TargetScan, miRanda, miRDB and miRWalk. TargetScan – only conserved sites with either 8 mer or 7 mer interaction are presented with high cumulative weight context++Score (http://targetscan.org/)[97]. miRanda program considers complimentarity between the 3′ UTR of the genes and seed region of the miRNA. It uses miRSVR scores which is a machine learning method for ranking by down regulation score (http://www.microrna.org)[98]. miRDB program predicts miRNA targets based on support vector machines and statistical model. Higher the predicted target score, higher is the specificity of the prediction (http://mirdb.org)[99]. miRWalk program compares miRNA binding sites from 12 existing miRNA-mRNA target prediction programmes (DIANA-microT v4.0, DIANA-microT-CDS, miRanda, mirBridge, miRDB v4.0, miRmap, miRNAMap, PicTar2, PITA, RNA22 v2, RNAhybrid2.1 and Targetscan6.2). It also searches miRNA targets with experimentally verified miRNA target genes. miRWalk algorithm compares central pairing sites between miRNA and the target genes (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk)[100]

| microRNA | Sequence | Target scan (cumulative weighted++Score) | miRanda (miRSVR score) | miRDB (target score) | miRWalk 2.0 (*P*-value) |
|---|---|---|---|---|---|
| miR-369-3p | AATAATACATGGTTGATCTTT | Proline-rich nuclear receptor coactivator 1 (–0.56) | Leukocyte immunoglobulin-like receptor pseudogene 2 (–8.32) | Microtubule-actin crosslinking factor 1 (84) | Zinc finger DHHC-type containing 15 (0.0018) |
| miR-98 | TGAGGTAGTAAGTTGTATTGTT | High mobility group AT-hook 2 (–2.67) | DNA replication helicase 2 homolog (–3.04) | SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily a, containing DEAD/H box 1 (100) | Leucine rich repeats and immunoglobulin like domains 2 (0.001) |
| miR-26b | TTCAAGTAATTCAGGATAGGT | Polymerase (RNA) III (DNA directed) polypeptide G (–1.02) | STE20-related kinase adaptor beta (–3.24) | Solute carrier family 2 (facilitated glucose transporter), member 13 (100) | No predicted target |
| MCMV-miR-M23-1-5p | CTCGGTACGGACGGGGAACCGT | Viral miRNA. No predicted targets found | | | |
| miR-1274a | Dead miRNA | | | | |
| miR-101b | TCGGTTATCATGGTACCGATGCT | Succinate dehydrogenase complex assembly factor 1 (–0.98) | Stanniocalcin 1 (–2.86) | Cysteine-rich hydrophobic domain 2 (82) | Zinc finger (CCCH type), RNA binding motif and serine/arginine rich 1 (0.0047) |
| let-7a | TGAGGTAGTAGGTTGTATAGTT | High mobility group AT-hook 2 (–2.67) | High mobility group AT-hook 2 (-2.69) | SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily a, containing DEAD/H box 1 (100) | Mitogen-activated protein kinase kinasekinase 1 (0.006) |
| let-7g | TGAGGTAGTAGTTTGTACAGTT | High mobility group AT-hook 2 (–2.67) | Chromosome 14 open reading frame 28 (–2.92) | Lin-28 homolog B (100) | Zinc finger protein 341 (0.000) |
| miR-193a-3p | AACTGGCCTACAAAGTCCCAGT | Interleukin 17 receptor D (–0.95) | Reticulon 4 interacting protein 1 (–1.18) | FH2 domain containing 1 (100) | ArfGAP with FG repeats 2 (0.000) |
| miR-192-3p | CTGCCAATTCCATAGGTCACAG | Chromosome 1 open reading frame 186 (–0.93) | ATPase, Ca++ transporting, type 2C, member 1 (–2.69) | RAB1A, member RAS oncogene family (99) | Itchy E3 ubiquitin protein ligase (0.0001) |

(*Contd*)

**Table 4.** (*Contd*)

| microRNA | Sequence | Target scan (cumulative weighted++Score) | miRanda (miRSVR score) | miRDB (target score) | miRWalk 2.0 (*P*-value) |
|---|---|---|---|---|---|
| miR-142-5p | CATAAAGTAGAAAGCACTACT | Zinc finger protein, FOG family member 2 (–0.84) | Sphingosine-1-phosphate phosphatase 1 (–4.33) | Zinc finger protein, FOG family member 2 (100) | ATPase Na+/K+ transporting subunit beta 1 (0.000) |
| miR-465d | TATTTAGAATGGTACTGATGTGA | Lipolysis stimulated lipoprotein receptor (–0.95) | | Vesicle-associated membrane protein 2 (99) | Rnaseh2a ribonuclease H2 (0.0001) |
| miR-677 | TTCAGTGATGATTAGCTTCTGA | POU class 5 homeobox 1B (–1.77) | Lysine (K)-specific demethylase 2A (–3.01) | Baculoviral IAP repeat-containing 6 (100) | SH3-domain kinase binding protein 1 (0.0010) |
| miR-694 | CTGAAAATGTTGCCTGAAG | Arylsulfatase family, member K (–0.66) | Family with sequence similarity 154, member B (–3.93) | Son DNA binding protein (100) | Dystrophin related protein 2 (0.0001) |
| miR-374* | GGTTGTATTATCATTGTCCGAG | Chromosome 5 open reading frame 28 (–0.67) | E74-like factor 1 (–4.64) | YY1 transcription factor (99) | AT rich interactive domain 2 (0.008) |
| miR-450b-5p | TTTTGCAGTATGTTCCTGAATA | Calcium/calmodulin-dependent protein kinase II inhibitor 1 (–0.78) | Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6 (–4.26) | RAB10, member RAS oncogene family (100) | Centromere protein J (0.000) |
| miR-464 | Dead miRNA | | | | |
| miR-377 | ATCACACAAAGGCAACTTTTGT | LIM homeobox transcription factor 1, alpha (–0.87) | Zinc finger protein 367 (–2.67) | Feline leukemia virus subgroup C cellular receptor 1 (100) | Mus musculus cyclin-dependent kinase 7 (0.0001) |
| miR-20a* | ACTGCATTACGAGCACTTAAAG | High mobility group nucleosomal binding domain 2 (–1.20) | Solute carrier family 38, member 2 (–3.40) | Solute carrier family 38, member 2 (100) | Nuclear factor of activated T cells 5 (0.000) |
| miR-466d-3p | TATACATACACGCACACATAG | chemokine (C-X-C motif) ligand 5 (–2.62) | EGF-like module containing, mucin-like, hormone receptor-like sequence 4 (–3.63) | Unkempt homolog (Drosophila) (100) | Zinc finger and BTB domain containing 42 (0.000) |
| miR-21 | TAGCTTATCAGACTGATGTTGA | Zinc finger protein 367 (–0.72) | Chloride channel accessory 3 (pseudogene) (–2.63) | Pellino E3 ubiquitin protein ligase 1 (99) | C-C motif chemokine receptor 7 (0.002) |
| miR-25 | CATTGCACTTGTCTCGGTCTGA | CD69 molecule (–1.77) | CD69 molecule (–3.67) | CD69 molecule (100) | Zinc finger protein 28 (0.0001) |

(*Contd*)

**Table 4.** (*Contd*)

| microRNA | Sequence | Target scan (cumulative weighted++Score) | miRanda (miRSVR score) | miRDB (target score) | miRWalk 2.0 (*P*-value) |
|---|---|---|---|---|---|
| let-7i | TGAGGTAGTAGTTTGTGCTGTT | High mobility group AT-hook 2 (-2.67) | High mobility group AT-hook 2 (-2.77) | SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily a, containing DEAD/H box 1 (100) | Zinc finger protein 341 (0.000) |
| miR-27a | TTCACAGTGGCTAAGTTCCGC | GRIP and coiled-coil domain containing 2 (-1.08) | Akirin 1 (–3.44) | Eyes absent homolog 4 (Drosophila) (100) | Cyclic nucleotide gated channel alpha 3 (0.0001) |
| miR-150 | TCTCCCAACCCTTGTACCAGTG | V-myb avian myeloblastosis viral oncogene homolog (–1.14) | V-mybmyeloblastosis viral oncogene homolog (avian) (–3.21) | Zinc finger and BTB domain containing 4 (98) | Mitochondrial amidoxime reducing component 1 (0.000) |
| miR-126 | CATTATTACTTTTGGTACGCG | plexin B2 (–0.58) | Homeobox A3 (–1.21) | KN motif and ankyrin repeat domains 2 (58) | TSC complex subunit 1 (0.0012) |
| miR-210 | CTGTGCGTGTGACAGCGGCTGA | Iron-sulphur cluster assembly enzyme (–0.93) | Apoptosis-inducing factor, mitochondrion-associated, 3 (–1.35) | Fibroblast growth factor receptor-like 1 (99) | Myocyte enhancer factor 2C (0.0010) |
| miR-155 | TTAATGCTAATTGTGATAGGGGT | Zinc finger protein 385D (–0.80) | ASF1 anti-silencing function 1 homolog A (-3.41) | WEE1 G2 checkpoint kinase (98) | Transcription factor 12 (0.0001) |
| miR-451 | AAACCGTTACCATTACTGAGTT | Odd-skipped related 1 (Drosophila) (–093) | Proteasome (prosome, macropain) subunit, beta type, 8 (-1.30) | HECT and RLD domain containing E3 ubiquitin protein ligase 3 (100) | TBC/LysM-associated domain containing 1 (0.0000) |
| miR-223 | CGTGTATTTGACAAGCTGAGTT | F-box and WD repeat domain containing 7, E3 ubiquitin protein ligase (-1.05) | F-box and WD repeat domain containing 7 (–3.67) | F-box and WD repeat domain containing 7, E3 ubiquitin protein ligase (100) | BCL2 associated transcription factor 1 (0.0003) |

factor c-myb that regulates lymphocyte development[69]. Thus upregulation of miR-150 leads to negative regulation of B and T cells and contributes directly to a fatal outcome by mounting an inefficient immune response (Tables 2 and 4).

## MicroRNAs in erythrocytic stage of malaria

It is well known that homozygous sickle cell erythrocytes (HbSS) are resistant to *Pf* infection when compared to normal homozygous haemoglobin carrying (HbAA) erythrocytes[70]. LaMonte *et al.*[71] studied the role of miRNAs in this protection phenomenon and established that miRNAs are the intrinsic factors that contribute to resistance towards *Pf* in homozygous (HbSS) or heterozygous sickle trait (HbAS) carrying erythrocytes[71]. They observed that during blood stage of *Pf* infection miR-451, let-7i and miR-223 translocate inside the parasite (Tables 2 and 4). MiR-451 and let-7i reduced the parasite growth by integrating into the parasite messenger RNA thereby disrupting ribosome loading, leading to translation inhibition[71]. Furthermore, LaMonte *et al.*[71] found that parasitized erythrocytes in HbAS or HbSS genotyped individuals were resistant to malaria through translation inhibition of some crucial parasite genes. It is interesting to note that although erythrocytes lack RNAi machinery and are enucleated and hence ineffective to alter miRNA levels, *Plasmodium* fails to survive in haemoglobin variant erythrocyte. It is argued that variant haemoglobin carrying erythrocytes due to sickling ability under low oxygen tension rupture and thus have short life span and are replaced by new erythrocytes faster than individuals carrying normal erythrocytes. These erythrocytes being fairly young may still contain residual miRNAs synthesized during nucleated reticulocyte and earlier stages that becomes available to interact with *Plasmodium* genes and integrates to block translation. The above presented evidences indicate that resistance to *Plasmodium* infection in HbAS/HbSS erythrocytes may be due to inability of *Plasmodium* to adopt itself to the molecular environment of low variant haemoglobin gene carrying erythrocytes.

## Non-coding RNAs in *Plasmodium*

Experimental evidences indicate absence of any transcription factors or RNAi machinery essential for miRNA-mediated gene regulation in *Pf*. However, silico screening of *Pf* genome by Mourier *et al.*[72] revealed 33 novel non-coding RNA (ncRNAs) transcripts expressed during erythrocytic phase of Pf growth cycle[72]. Interestingly, presence of ncRNAs in *Pf* genome suggests gene regulation by unknown mechanism unique to *Plasmodium* spp. Identification of novel ncRNAs in erythrocytic stage of *Pf* genome indicates that more ncRNAs with critical roles will be identified in other stages of growth in *Pf* genome. Due to lack of functional studies, cellular roles of these ncRNAs remain unknown.

Of note, transactive response (TAR) DNA binding protein-43 (TDP-43) is known to be a component of Drosha complex, suggesting their involvement in miRNA processing[73]. Structurally, TDP-43 closely resembles heterogenous ribonucleoproteins (hnRNPs) and is intricately involved in RNA biogenesis through regulation of transcription, splicing, transport and translation[74,75.] TDP-43 contains two RNA recognition motifs (RRMs), viz. RRM1 and RRM2 (ref. 77). RRM1 of TDP-43 is responsible for binding to single stranded RNA with structural specificity of minimum penta UG repeats[76]. TDP-43 is also involved in RNA transport through nucleo-cytoplasmic shuttling[77]. We looked for TDP-43 homologs in *Pf* and found PF3D7_0414500 with 42% identity and conserved RRM1 and RRM2 domains. Presence of TDP-43 homolog in *Pf* proteome indicates that *Pf*-mediated manipulation of host RNA expression could be through multiple mechanisms. The ability of TDP-43 to bind single stranded RNA and their nucleo-cytoplasmic shuttling indicates that miRNA translocation into PV could be mediated via TDP-43. Furthermore, role of TDP-43 in miRNA maturation and structural specificity explains selective transport and selective dysregulation of certain miRNAs.

Although it is well-known that *Plasmodium* lacks a functional RNAi machinery, malaria parasite metabolic pathway database (PlasmoDB) indicate links of TDP-43 with P-body. Additionally, it also revealed the presence of numerous human homologs of effector proteins involved in mRNA decay such as mRNA decapping enzyme (PF3D7_1032100), exoribonuclease (PF3D7_0909400), etc. It is interesting to note that mRNA decay is one of the mechanisms through which miRNA degrades/suppresses mRNA translation. So it is unclear why proteins involved in mRNA decay process are expressed in *Pf* when RNAi process itself is lacking in *Plasmodium*. So far, there is no experimental evidence to support our bioinformatic findings.

## Conclusion

The present review discussed the role of the RNAi in pathogenesis through disruption of cellular homeostasis. Here studies listed have shown response caused by various stresses. Studies investigating crucial roles of miRNAs are lacking, yet essential to arrive at conclusive inferences.

Investigation on miRNA expression in *Plasmodium* infection report a non-overlapping miRNA signature indicating variable response to different *Plasmodium* strains rather than a generalized response. However, indirect evidences show that *Plasmodium* invasion alters

miRNA profile in such a way that it dampens host immune response necessary to eliminate parasites. Further, studies have revealed that parasitic invasion alters miRNA expression that negatively regulates activation, differentiation and proliferation of B and T cells, inflammatory cytokines and signalling pathways.

To summarize, we have presented three key phenomena observed during *Plasmodium* infection. MiRNAs are able to pass parasitophorus membrane. They act by both canonical and non-canonical mechanisms. *Plasmodium* manipulates miRNAs to ensure its protein synthesis and growth while suppressing host immune response and preventing host cell apoptosis through modulation of cell signalling pathways.

Malaria being a third world disease is relatively less studied, the problem is further compounded by technical challenges to study role of microRNAs during malaria. Initial challenges include difficulty to obtain contamination-free actively replicating parasites. Further *P. falciparum* blood-stage infection in chimeric mice generates around 1% parasitemia which is not sufficient to study. Thus numerous infected mice are required to obtain sufficient amount of parasites for the study. Since malaria has hepatic and blood stage, different strains of parasite are required to study the stages in animal models. Further it is difficult to establish a precise and efficient cerebral malarial model in animals that could efficiently mimic complexity of human host. Besides these, studying miRNAs has its own technical challenges. miRNAs constitute only a small fraction of the total RNA pool, thus extracting miRNA in pure form is difficult. The problem is further aggravated by the fact that *Plasmodium* parasites shed out RNA and proteins as extracellular vesicles called exosomes. Further it is difficult to prevent miRNA degradation by RNases. As most miRNAs are identified bioinformatically, they need to be experimentally validated for their expression. Thereafter target gene needs to be identified. Since an miRNA can target multiple mRNAs and multiple miRNAs can target single mRNAs, experimental validation of miRNA–mRNA interaction could lead to misleading results. Lastly in the absence of stable and verified endogenous controls it is difficult to normalize the miRNA data.

Interestingly, the presence of ncRNAs in *Pf* may play a crucial role in gene regulation either by hijacking host RNAi machinery or by employing a novel mechanism unique to *Plasmodium*. Lastly, there are ample indirect evidences that point to the fact that *Plasmodium* spp. manipulates host RNAi machinery. This interaction with host RNAi machinery and/or its components needs to be proven experimentally to arrive at conclusive evidence to implicate *Plasmodium* of the alleged manipulation.

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Received 4 August 2016; revised accepted 7 August 2018

doi: 10.18520/cs/v115/i12/2219-2231