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Natural antisense transcripts in *Plasmodium falciparum* isolates from patients with complicated malaria

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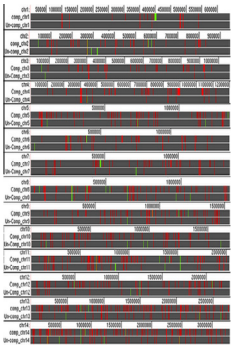
HIGHLIGHTS

- This is the first report on NATs in *Plasmodium falciparum* from patient isolates.
- A total of 797 NATs have been detected of which 545 are unique to this study.
- The NATs map to a broad range of biochemical/ metabolic pathways.
- The majority of NATs were positively correlated with its sense counterpart.
- Complication specific change in antisense/sense transcripts ratio was observed.

GRAPHICAL ABSTRACT



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ABSTRACT

Mechanisms regulating gene expression in malaria parasites are not well understood. Little is known about how the parasite regulates its gene expression during transition from one developmental stage to another and in response to various environmental conditions. Parasites in a diseased host face environments which differ from the static, well adapted *in vitro* conditions. Parasites thus need to adapt quickly and effectively to these conditions by establishing transcriptional states which are best suited for better survival. With the discovery of natural antisense transcripts (NATs) in this parasite and considering the

Abbreviations: NATs, natural antisense transcripts; IDC, intra erythrocytic developmental cycle; PFC, *Plasmodium falciparum* complicated samples; PFU, *Plasmodium falciparum* uncomplicated samples; HS-LAS, high sense and low antisense transcripts; HAS-LS, high antisense and low sense transcripts; NC-AS/S, no change in antisense and sense transcript ratio; Glc, glucose; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; DF, D-fructose; F1P, fructose-1-phosphate; F1,6BP, fructose-1,6-bisphosphate; DHAP, dihydroxy-acetone-phospahte; GADP, glyceraldehydes-3-phosphate; 1,3 BPG, 1,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglucerate; PEP, phosphoenolpyruvate; Pyr, pyruvate; Lac, lactate; Ac-CoA, acetyl-CoA; GlycP, glycerol-3-phosphate; Glyc, glycerol; Man, mannose; Man6P, mannose-6-phosphate; Man1P, mannose-1-phosphate; GDP-Man, GDP-mannose; GlcN, glucosamine; GlcN6P, glucosamine-6-phosphate; GlcNAc6P, N-acetyl-glucosamime-6-phosphate; GlcNAc1P, N-acetyl-glucosamine-1-phosphate; UDP-GlcNAc, UDP-N-acetyl-glucosamine; 6PGL, 6-phosphoglucono-δ-lactone; 6PGa, 6-phosphogluconate; Ru5P, ribulose-5-phosphate; R5P, ribose-5-phosphate; X5P, xylulose-5-phosphate; S7P, sedoheptulose-7-phosphate; E4P, erythrose-4-phosphate; PRPP, phosphoribosylpyrophosphate; Asp, aspartate; Asn, aspargine; NcASP, N-carbamoyl-L-aspartate; Gln, glutamine; Glu, glutamate; 2OG, 2-oxoglutarate; ICT, isocitrate; Cit, citrate; OAA, oxaloacetate; Mal, malate; Suc-CoA, succinyl CoA; Suc, succinate; Fum, fumarate; GPI, glycophosphatidylinositol; ACON-C, cis-aconitate.

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various proposed mechanisms by which NATs might regulate gene expression, it has been speculated that these might be playing a critical role in gene regulation. We report here the diversity of NATs in this parasite, using isolates taken directly from patients with differing clinical symptoms caused by malaria infection. Using a custom designed strand specific whole genome microarray, a total of 797 NATs targeted against annotated loci have been detected. Out of these, 545 NATs are unique to this study. The majority of NATs were positively correlated with the expression pattern of the sense transcript. However, 96 genes showed a change in sense/antisense ratio on comparison between uncomplicated and complicated disease conditions. The antisense transcripts map to a broad range of biochemical/metabolic pathways, especially pathways pertaining to the central carbon metabolism and stress related pathways. Our data strongly suggests that a large group of NATs detected here are unannotated transcription units antisense to annotated gene models. The results reveal a previously unknown set of NATs that prevails in this parasite, their differential regulation in disease conditions and mapping to functionally well annotated genes. The results detailed here call for studies to deduce the possible mechanism of action of NATs, which would further help in understanding the *in vivo* pathological adaptations of these parasites.

1. Introduction

Malaria caused by protozoan parasites of the genus *Plasmodium,* accounts for 243 million cases of infection and 8,63,000 deaths world-wide per annum (Mendis et al., 2009). Five species of *Plasmodium* can infect humans, of which *Plasmodium falciparum* is considered the most virulent and responsible for much of the mortality due to malaria. The challenges in fighting malaria include its complex lifecycle and widespread development of drug resistance. *P. falciparum* completes its life cycle in two hosts: the vertebrate host (Human) and the invertebrate host (Mosquito). In the human host it passes through two stages: The exo-erythrocytic stage and the intra erythrocytic stage. In the intra erythrocytic developmental stage/cycle (IDC), *Plasmodium* infects the red blood cells (RBCs), morphs through ring, trophozoite and schizont stages and emerges by rupturing the RBCs (Llinas and DeRisi, 2004). Microarray and proteomics based data has revealed significant regulation of transcripts and protein expression profiles associated with these morphological and developmental changes during the various stages of its life cycle (Bozdech et al., 2003a,b; Le Roch et al., 2004; Le Roch et al., 2003). Remarkable changes in the transcript abundance of the *P. falciparum* transcriptome were observed when subjected to different perturbed conditions (Natalang et al., 2008; Oakley et al., 2007; Tamez et al., 2008). Transcriptional variation has also been documented between the culture adapted field isolates and long-term laboratory-adapted isolates (Mackinnon et al., 2009). *In vivo* expression profiles of the *P. falciparum* parasite derived directly from blood samples of infected patients showed distinct transcriptional states (Daily et al., 2007).

The molecular mechanisms underlying this pattern of transcriptional regulation are not well understood (Painter et al., 2011). The number of conserved transcription factors annotated in this parasite is extremely low. This has been partially compensated by the discovery of a large Apicomplexan AP2 (ApiAP2) protein family containing Apitela2 (AP2) domains (Balaji et al., 2005). There are many other possibilities of how gene regulation might happen in this parasite like controls at transcriptional (Cui and Miao, 2010; Painter et al., 2011), post transcriptional (Shock et al., 2007) and/or at translational level (Mair et al., 2006). Gene regulation could also be due to natural antisense transcripts (NATs), which may influence gene expression patterns (Militello et al., 2008).

NATs are transcribed from the strand opposite to the template DNA strand and may hybridize with the sense transcripts of same genomic loci (cis-NATS) or to the complementary transcripts of separate genomic loci (trans-NATs). This has been implicated in regulating gene expression in both prokaryotes and eukaryotes through diverse postulated mechanisms (Faghihi and Wahlestedt, 2009; Werner and Berdal, 2005; Lavorgna et al., 2004; Rogozin et al., 2002; Wagner and Simons, 1994). Gene expression regulation by NATs in different organisms includes genomic imprinting, transcriptional collision, X chromosome inactivation, alternative splicing and termination, RNA interference, translational regulation, and RNA editing (Faghihi and Wahlestedt, 2009; Lavorgna et al., 2004).

Presence of antisense transcripts in *P. falciparum* transcriptome was first reported from SAGE data (Gunasekera et al., 2004; Patankar et al., 2001). Further evidence supporting the presence of antisense transcripts have come from different groups using various approaches like microarrays, nuclear run-on experiments, cDNA sequencing, and RNA-sequencing (Lopez-Barragan et al., 2011; Lu et al., 2007; Militello et al., 2005; Raabe et al., 2010; Ralph et al., 2005; Sorber et al., 2011). It has been speculated that NATs might be playing an important role in regulating gene expression in this species as evident from antisense transcripts detected from various developmental stages of the parasites. NATs have been reported to be synthesized by RNA polymerase II (Militello et al., 2005). The diversity of NATs reported till date is from laboratory-adapted isolates. Prevalence and abundance of NATs in *in vivo* disease conditions caused by the parasite has not been detailed as yet.

In this study, we report the prevalence of natural antisense transcripts in *P. falciparum* clinical isolates from patients showing uncomplicated and complicated disease conditions on a genome wide scale using custom designed strand specific microarray. Their expression pattern, genome-wide distribution and differences in prevalence under differing clinical conditions have been explored. Functional analysis has been performed to try and understand the probable biological role. Finally, the data has been compared with the previously published data from *in vitro* culture conditions. To the best of our knowledge this is the first report of the presence of NATs in *P. falciparum* infections causing complicated disease conditions.

2. Materials and methods

2.1. 244K custom array design

P. falciparum 244K gene expression array was designed on Agilent platform with probes having 60-mer oligonucleotides representing the 3D7 transcript sequences (PlasmoDB version 5.3) (Aurrecoechea et al., 2009; Gardner et al., 2002), NCBI EST sequences of *P. falciparum* and apicoplast sequences of *P. falciparum* (Wilson et al., 1996) and *P. vivax* (Saxena et al., 2012). Out of 5532 *P. falciparum* transcript sequences available in the PlasmoDB (Release 5.3) (Aurrecoechea et al., 2009; Gardner et al., 2002), probes were designed with an average of 8 probes per sequence