Infection, Genetics and Evolution 20 (2013) 428–443

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Revealing natural antisense transcripts from *Plasmodium vivax* isolates: Evidence of genome regulation in complicated malaria

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ARTICLE INFO

Article history:
Received 31 May 2013
Received in revised form 24 September 2013
Accepted 25 September 2013
Available online 11 October 2013

Keywords:
Natural antisense transcripts
Strand specific microarray
Plasmodium vivax
Uncomplicated malaria
Complicated malaria

ABSTRACT

Plasmodium vivax is the most geographically widespread human malaria parasite causing approximately 130–435 million infections annually. It is an economic burden in many parts of the world and poses a public health challenge along with the other *Plasmodium* sp. The biology of this parasite is less studied and poorly understood, in spite of these facts. Emerging evidence of severe complications due to infections by this parasite provides an impetus to focus research on the same. Investigating the parasite directly from infected patients is the best way to study its biology and pathogenic mechanisms. Gene expression studies of this parasite directly obtained from the patients has provided evidence of gene regulation resulting in varying amount of transcript levels in the different blood stages. The mechanisms regulating gene expression in malaria parasites are not well understood. Discovery of Natural Antisense Transcripts (NATs) in *Plasmodium falciparum* has suggested that these might play an important role in regulating gene expression. We report here the genome-wide occurrence of NATs in *P. vivax* parasites from patients with differing clinical symptoms. A total of 1348 NATs against annotated gene loci have been detected using a custom designed microarray with strand specific probes. Majority of NATs identified from this study shows positive correlation with the expression pattern of the sense (S) transcript. Our data also shows condition specific expression patterns of varying S and antisense (AS) transcript levels. Genes with AS transcripts enrich to various biological processes. To our knowledge this is the first report on the presence of NATs from *P. vivax* obtained from infected patients with different disease complications. The data suggests differential regulation of gene expression in diverse clinical conditions, as shown by differing sense/antisense ratios and would lead to future detailed investigations of gene regulation.

Abbreviations: GLC, glucose; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; FBP, fructose 1,6-bis phosphate; DHAP, dihydroxy-acetone-phosphate; GAP, glyceraldehyde 3-phosphate; 1,3 BPG, 1,3-biphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phophoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; LAC, lactate; 6PGNL, 6-phosphoglucono-δ-lactone, 6PGNT, 6-phophogluconate; 6PGNT, 6-phophogluconate; RU5P, ribulose-5-phosphate; X5P, xylulose-5-phosphate, G3P, glyceraldehyde-3-phophate; G3P, glyceraldehyde-3-phophate; E4P, erythrose-4-phosphate; R5P, ribose-5-phosphate; S7P, sedoheptulose-7-phosphate, F6P, fructose-6-phosphate; F6P, fructose-6-phosphate; DR1P, deoxyribose-1-phosphate; DR5P, deoxyribose-5-phosphate; AC-COA, acetyl-CoA; CIT, citrate; ACON-C, cis-aconitate; ICT, Isocitrate; 2OG, 2-Oxaloglutarate; SUC-COA, succinyl-COA; SUC, succinate; FUM, fumarate; MAL, malate; OXA, oxaloacetate; ASP, aspartate; GLYC3P, glycerol-3-phosphate; GLYC, glycerol; GLN, glutamine; GLU, glutamate; MAN, mannose; MAN6P, mannose-6-phosphate; MAN1P, mannose-1-phosphate; GDP-MAN, GDP-Mannose; GDP-ODM, GDP-4-oxo-6-deoxy-mannose; GDP-FUC, GDP-Fucose; GLCN6P, glucosamine-6-phosphate; GLCNAC6P, N-acetyl-glucosamime-6-phosphate; GLCNAC1P, N-acetyl-glucosamine-1-phosphate; UDP-GLCNAC, UDP-N-acetyl-glucosamine. Gene coding for protein:PVX_114315, hexokinase, putative; PVX_118255, fructose 1,6-bisphosphate aldolase; PVX_118495, triosephosphate isomerase; PVX_099535, phosphoglycerate kinase; PVX_080515, ribose 5-phosphate epimerase; PVX_001945, deoxyribose-phosphate aldolase; PVX_094915, GDP-L-fucose synthetase; PVX_111455, glucosamine-fructose-6-phosphate aminotransferase; PVX_091100, succinyl-CoA synthetase alpha subunit.

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http://dx.doi.org/10.1016/j.meegid.2013.09.026

1. Introduction

The protozoan parasite *Plasmodium vivax* is considered the most widely distributed of the human malarial parasites. It is conservatively estimated that there are 2.85 billion people at risk and 130–435 million infections annually (Guerra et al., 2010; Hay et al., 2004). Over 50% of malaria cases caused by this species occur in India and thus makes her the major contributing country to the worldwide burden of *P. vivax* (Joshi et al., 2008; Kumar et al., 2007). *P. vivax* is still primarily considered to be the cause of benign tertian malaria; however, recent studies have documented the existence of patients with complications pathologically similar to those seen in severe *Plasmodium falciparum* malaria (Alexandre et al., 2010; Kochar et al., 2009, 2007, 2005). Very little is known about molecular mechanisms involved in complicated *P. vivax* malaria. Disease severity has been associated with increased expression of drug resistance genes (Fernandez-Becerra et al., 2009; Tjitra et al., 2008). Studies have also shown that *P. vivax* infected RBC (*iRBC*) can adhere to placental Glycosaminoglycans (Chotivanich et al., 2012) and endothelial receptors like ICAM-1 (Bernabeu et al., 2012 and Carvalho et al., 2010) which might contribute to the pathogenesis of *vivax* malaria.

Whole genome transcriptome studies from *P. vivax* using high density microarrays have provided a glimpse into the transcriptional states of blood stages in short term culture and stage specific expression patterns have been postulated (Bozdech et al., 2008). Evidence of gene regulation giving rise to varying amounts of transcript has been reported (Westenberger et al., 2010). Mechanisms of transcriptional regulation as in case of *P. falciparum* have also been postulated (Bozdech et al., 2008; Westenberger et al., 2010).

Most eukaryotic genomes produce non-coding RNAs (ncRNAs) which are considered to have functional role in gene regulation (Matrajt, 2010; Moreno Díaz de la Espina et al., 2005; Morey and Avner, 2004). Natural antisense transcripts (NATs) are ncRNAs which are complementary to their sense counterpart and could be from the same locus (cis-NATs) or from different loci (trans-NATs). Studies from humans, mouse, drosophila, fungi and yeast suggest that the expressions of these regulatory ncRNAs occur during specific stages of development or in response to nutritional or environmental conditions (Donaldson and Saville, 2012). Genome-wide antisense transcripts have been described in asexual and sexual stages of *P. falciparum* by various techniques (Gunasekera et al., 2004; Lopez-Barragan et al., 2011; Patankar et al., 2001; Raabe et al., 2010; Sorber et al., 2011). Studies from our laboratory show the presence and differential expression of NATs in *P. falciparum* from patients showing complicated and un-complicated disease pathogenesis (Communicated).

The study detailed here documents, for the first time, the presence of genome-wide NATs from *P. vivax* RNA isolated from patients showing complicated and uncomplicated disease pathologies. The presence of antisense transcripts has been validated by strand specific reverse transcriptase (RT) PCR for a subset of genes. In addition to documenting the genome-wide distribution of NATs, our results also indicate significant differences in distribution pattern and expression ratio profiles of NATs detected under these differing clinical conditions. This change in expression ratio in different clinical conditions has also been seen in a subset of genes using strand specific quantitative real-time PCR. Functional analysis has subsequently been performed to understand the possible biological roles.

2. Materials and methods

2.1. Study population and blood sample collection

Venous blood samples were collected (~5 ml) from 8 *P. vivax* infected adult patients admitted to S.P. Medical College, Bikaner, India during August to September, 2007. The patients exhibited symptoms categorized as uncomplicated or complicated malaria. The patient samples were collected on informed consent by the team of clinicians at S.P. Medical College, Bikaner, India according to hospital guidelines. Infection with *P. vivax* was confirmed by slide microscopy and RDTs (OptiMal test; Diamed AG, Cressier sur Morat, Switzerland, Falcivax test; Zephyr Biomedical System, Goa, India) in the hospital. Blood was immediately (within 15 min of collection) subjected to density gradient based separation (Histopaque 1077, Sigma Aldrich, USA) to separate the peripheral blood mononuclear cells (PBMCs) from the infected and uninfected erythrocytes following manufacturer's instructions. Both fractions were washed with phosphate buffered saline (PBS) twice and lysed using 4 volume of Tri-Reagent (Sigma Aldrich, USA) and stored at −80 °C. Subsequently, samples were transported in cold chain to BITS, processed and evaluated by 18S rRNA based multiplex PCR and 28S rRNA based nested PCR, to rule out any possibility of *P. falciparum* co-infection as mentioned (Das et al., 1995; Kochar et al., 2005; Pakalapati et al., 2013a,b). All laboratory investigations to rule out possibility of any other possible cause for symptoms exhibited (complicated *P. vivax* cases) were done as described by Kochar et al. (2009). Criteria for determination of complicated disease were based on World Health Organization (WHO) guidelines (WHO, 2010). Clinical profile of all the patients included in this study is presented in the Table 1. All hepatic dysfunction cases had serum bilirubin >3 mg/dL, all renal failure cases had serum creatinine >3 mg/dL, all thrombocytopenia cases had platelets <100,000/mm^3 of blood and all anemia cases had blood hemoglobin level $\leqslant$7 g/dL.

2.2. RNA sample preparation

Total RNA and DNA were isolated from samples using the manufacturer's protocol (Tri-Reagent, Sigma Aldrich, USA). All RNA samples were processed on denaturing agarose gel and found to be intact. Total RNA integrity was also assessed using RNA 6000 Nano Lab Chip on the 2100 Bioanalyzer (Agilent, Palo Alto, CA) following the manufacturer's protocol. Total RNA purity was assessed by the NanoDrop® ND-1000 UV–Vis Spectrophotometer (Nanodrop technologies, Rockland, USA).

2.3. Labeling

The one uncomplicated *P. vivax* RNA sample (PVU) and the seven complicated parasite samples (pooled) (PVC) were labeled in equimolar amounts. The labeling was performed using Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies) as described by the manufacturer at Agilent certified service lab of Genotypic Technology, Bangalore.

2.4. Hybridization and scanning

The labeled cRNA samples were hybridized to the 244K array (designed by Genotypic Technology, Bangalore, India in collaboration with BITS Pilani, Pilani, India exclusively for this study) with Gene Expression Hybridization Kit (Agilent Technologies, Part No. 5188-5242). Hybridization was carried out in Agilent's Surehyb Chambers at 65 °C for 16 h. The hybridized slides were washed using Gene Expression wash buffers (Agilent Technologies, Part No. 5188-5327) and scanned using the Agilent Microarray Scanner (Agilent, Palo Alto, CA, G Model G2565BA) at 5 μm resolution. Data extraction from images was done using Feature Extraction software (Agilent Technologies).