Disruption of Plasmodium falciparum histidine-rich protein II may affect haem metabolism in the blood stage

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Abstract

Background: Haem is a key metabolic factor in the life cycle of the malaria parasite. In the blood stage, the parasite acquires host haemoglobin to generate amino acids for protein synthesis and the by-product haem for metabolic use. The malaria parasite can also synthesize haem *de novo* by itself. *Plasmodium falciparum*-specific histidine-rich protein 2 (*Pf*HRP2) has a haem-binding site to mediate the formation of haemozoin, a biocrystallized form of haem aggregates. Notably, the gene regulates the mechanism of haemoglobin-derived haem metabolism and the *de novo* haem biosynthetic pathway in the *Pfhrp2*-disrupted parasite line during the intraerythrocytic stages.

Methods: The CRISPR/Cas9 system was used to disrupt the gene locus of *Pfhrp2*. DNA was extracted from the transgenic parasite, and polymerase chain reaction (PCR), Southern blotting and Western blotting were used to confirm the establishment of transgenic parasites. RNA-Seq and comparative transcriptome analysis were performed to identify differences in gene expression between 3D7 and *Pfhrp2*$^-$ 3D7 parasites.

Results: *Pfhrp2*$^-$ transgenic parasites were successfully established by the CRISPR/Cas9 system. A total of 964, 1261, 3138, 1064, 2512, and 1778 differentially expressed genes (DEGs) were identified in the six comparison groups, and a total of 373, 520, 1499, 353, 1253, and 742 of the DEGs were upregulated, and 591, 741, 1639, 711, 1259, and 1036 of the DEGs were downregulated, respectively. Five DEGs related to haem metabolism and synthesis were identified in the comparison groups of six time points (0, 8, 16, 24, 32, and 40 h after merozoite invasion). The genes encoding ALAS and FC, related to haem biosynthesis, were found to be significantly upregulated in the comparison groups, and the HO, SPP, and PBGD genes were found to be significantly downregulated. No GO terms were significantly enriched in haem-related processes (Q value=1).

Conclusion: Our data revealed changes in the transcriptome expression profile of the *Pfhrp2*$^-$3D7 parasite during the intraerythrocytic stages. The above findings provide insight at the gene transcript level for further research and development of anti-malaria drugs.

Introduction

Malaria remains one of the most significant health challenges for human beings. *Plasmodium falciparum* is one of the deadliest and most burdensome human malaria parasites and caused approximately 228 million cases of malaria, resulting in 405 000 deaths, worldwide in 2018 [1].

Haem is a crucial metabolic factor that is derived primarily from the parasite's haem biosynthesis pathway [2] at the early ring stage and from haemoglobin digestion at later stages [3]. Malaria parasites ingest more than 75% of the host cell haemoglobin in a short period due to their nutritional requirements in the blood stage [3, 4]. The haemoglobin is digested in the food vacuole to generate amino acids,

releasing the toxic haem moiety [5]. Since haem is toxic, excess haem is stored as the pigment haemozoin, a biocrystallized form of haem aggregates [6].

P. falciparum-specific histidine-rich protein 2 (*Pf*HRP2, PlasmoDB: PF3D7_0831800, www.plasmodb.org) constitutes two exons, and it is a water-soluble protein that is released from infected erythrocytes and circulated in malaria-infected patients [7, 8]. HRPII and HRP III, as homologous proteins, could bind haem in the digestive vacuole and play a role in haemozoin formation [9-11]. Chloroquine binds to toxic haem metabolites and thereby prevents their conversion to and deposition as the inert haemozoin [12]. The malaria parasite also has a haem biosynthetic pathway to acquire haem. Studies with *Plasmodium berghei*-infected mice and *P. falciparum* in cultures delta-aminolevulinic acid synthetase (ALAS) and ferrochelatase (FC) knockout (KO) parasites have indicated that the haem biosynthetic pathway is nonessential for parasite survival in the blood stages [13, 14].

The clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system has been successfully used in genome editing of the human malaria parasites *P. falciparum* and *Plasmodium yoelii* [15-17]. Briefly, a single-guide RNA (sgRNA) guides the Cas9 endonuclease to cause double-strand breaks (DSBs), and DSBs can be repaired by homologous recombination using donor DNA. Transgenic parasites can be obtained after 3-6 weeks [15, 16]. This has been demonstrated to be a highly precise and efficient method for genome editing.

The present study aimed to specifically disrupt the gene locus of *Pfhrp2* in the wild-type 3D7 parasite and investigate how explicit gene disruption affects haemoglobin-derived haem metabolism and the haem biosynthetic pathway at the gene level.

Methods

Parasite culture, synchronization, and pellet collection

P. falciparum (3D7 strain) cells at the asexual stage were cultured in vitro in human erythrocytes (blood group O+) obtained from the Beijing Red Cross Blood Center. The parasite was grown under 5% O_2 and 5% CO_2 in RPMI-1640 medium supplemented with 5 g/L Albumax II (Life Technologies), 2 g/L sodium bicarbonate, 25 mM HEPES (pH 7.4, adjusted with potassium hydroxide), 1 mM hypoxanthine, and 50 mg/L gentamicin as previously described [18].

For synchronization, parasite cells were cultured to at least 10% parasitaemia in T-75 flasks containing 50 ml of medium at 1% haematocrit. Then, the cells were moved from the flask to a 50-mL tube and centrifuged for 5 min at 500×g, and the supernatant was removed. Fifteen millilitres of 5% D-sorbitol solution was added to the pellet, followed by incubation at 37°C for 10 min, centrifugation, and removal of the supernatant. The culture was synchronized with three rounds of sorbitol treatment. Then, the invasion culture solution was collected at 8 h, 16 h, 24 h, 32 h, 40 h, and 46/0 h; 3

replicates were collected at each time point. A total of thirty-six samples were collected and centrifuged, and pellets were stored at -80°C until use.

Plasmid constructs and *Plasmodium* transfection

Based on the pUF1-Cas9 and pL6CS plasmids kindly provided by Jose-Juan Lopez-Rubio, pUF1-BSD-Cas9 and pL6CS-hDHFR-*hrp2* were constructed to disrupt the *Pfhrp2* locus (Fig. 1). Construction of the two plasmids and *Plasmodium* transfection were performed as described previously [19].

Briefly, pUF1-BSD-Cas9 expresses Cas9 nuclease and blasticidin S deaminase (BSD). The pL6CS-hDHFR-*hrp2* plasmid expresses donor DNAs and sgRNAs targeting the *Pf*hrp2 gene (guide^{hrp2}), and its left and right homologous arms were amplified separately by PCR from the genomic DNA of *P. falciparum* 3D7 (primers P1/P2 for the left arm and P3/P4 for right arm). The left homologous arm of hrp2 was ligated between the ASCI and AfLII sites of the pL6CS plasmid. The right homologous arm was ligated between the HindIII and EcoRI sites of the plasmid. The right homologous arm also used as a stop codon for the gene encoding hDHFR. The annealing reaction solution consisted of 1 μL of forward oligo (100 μM), 1 μL of reverse oligo (100 μM), and 23 μL of ddH_2O. The annealing conditions for the sgRNA were as follows: 2 cycles of 30 s at 94°C and 30 s at 72°C, followed by 30 s at 60°C, 30 s at 25°C, and 30 s at 16°C. Then, the annealed sgRNA was ligated into the pL6CS plasmid.

The transitional construct pL6CS-hDHFR-*hrp2* was transformed into competent cells, and plasmids were extracted and checked with restriction enzyme digestion and sequencing. After the correct transitionalpL6CS-hDHFR-*hrp2* construct was obtained, this transitional plasmid was linearized with *AvrII* and *XhoI*. The sgRNAs of *hrp2* were annealed and inserted into linearized transitional-pL6CS-hDHFR-*hrp2* plasmid using the In-Fusion kit. The construct was transformed into competent cells again and extracted. The final plasmid was verified by restriction enzyme digestion and DNA sequencing. The confirmed plasmid was isolated and used for electroporation to generate transgenic *P. falciparum* strains.

Electroporation of the two plasmids was carried out by the spontaneous uptake method using ~50 μg of maxi-prepped plasmid DNA and 8 square wave electroporation pulses of 356 V for 1 ms each, separated by 0.1 s. Drugs (final concentration of 2.5 mg/L for blasticidin S and 5 nmol/L for WR99210) were added into the complete medium post-transfection to kill the parasites that lacked episomal pUF1-BSD-Cas9 and pL6CS-hDHFR-*hrp2*. The location of sgRNA in the *hrp2* and gene disruption schematic are listed in Additional file 1: Figure S1. All primer and sgRNA sequences used for constructing plasmids are listed in Additional file 2: Table S1 and Additional file 3: Table S2.

Confirmation of successful transgene introduction via PCR

Twenty days after electroporation, live *P. falciparum* cells appeared, and genomic DNA was extracted from harvested parasite pellets using the Qiagen DNA Extraction Kit (Qiagen, Valencia, California USA). PCR was performed in a total volume of 20 μL, consisting of 10× buffer with 15 nM $MgCl_2$, 200 μM dNTPs, 15 μM forward and reverse primers (P1/P2, as indicated in Fig. 2), 0.69 units of *Taq* DNA

polymerase, and 2 μL of DNA template. An in vitro *P. falciparum* 3D7 culture was used as a positive control for *Pfhrp2* gene amplification experiments. All PCR products were separated and visualized on agarose gels, and products with the expected size were submitted for sequencing for further confirmation.

Southern blotting analysis

Genomic DNA was isolated from transgenic parasites as described above. In total, 5 μg of the parasite genomic DNA was digested overnight using *PstI* or *SacI* restriction endonuclease (TaKaRa Bio). The DNA products were separated on a 1.0% agarose gel and transferred to a Hybond^{TM} –N^{+} membrane (GE Healthcare Amersham^{TM}) using the high-salt capillary transfer method. Probes were PCR amplified, cleaned, and labelled with DIG-dUTP using the PCR DIG Probe Synthesis Kit (Roche). The blots were hybridized with the labelled probes, washed, and exposed to film and visualized in a cassette. All primer used for southern blotting analysis are listed in Additional file 4: Table S3.

Western blotting analysis

Successfully generated transgenic parasites were cultured in flasks. For the analysis of Pfhrp2 expression, when parasitaemia exceeded 5%, infected red blood cells (iRBCs) were collected and incubated with 0.15% saponin lysis solution on ice for 7 min. After centrifugation at 500×g for 5 min at room temperature (RT), the supernatants were collected and added to an appropriate amount of SDS-PAGE sample buffer, denatured at 95°C for 8 min, resolved by electrophoresis in a 12.5% polyacrylamide gel (Life Technologies) and transferred onto a 0.2-μm polyvinylidene difluoride (PVDF) membrane (Hybond LFP; GE Healthcare). HRP2 was specifically detected by using an anti-*Plasmodium falciparum* monoclonal antibody [MPFG-55P] (horseradish peroxidaseP) (Abcam, Massachusetts, USA).

Total RNA extraction

Total RNA was extracted using TRIzol according to the manufacturer's protocol. Briefly, pellets collected at different times from transgenic parasites were ground into powder by liquid nitrogen and transferred into a new tube with TRIzol reagent. The mix was shaken and kept for 5 min at RT and then centrifuged at 10,000×g for 5 min at 4°C. Chloroform/isoamyl alcohol (24:1) was added to the supernatant with lysis reagents. After centrifugation at 10,000×g for 10 min at 4°C, the supernatant was transferred into a new tube with an equal volume of isopropanol and kept at -20°C for 1 h. After centrifugation at 13,600×g for 20 min at 4°C, the supernatant was precipitated by ethanol and dried for 3 min. The RNA pellet was dissolved with RNase-free water.

mRNA library construction and sequencing

Oligo (dT)-attached magnetic beads were used to purify mRNA from parasite pellets. Purified mRNA was fragmented into small pieces with buffer at the appropriate temperature. First-strand cDNA was generated using random hexamer-primed reverse transcription, followed by second-strand cDNA

synthesis. Then, A-tailing mix and RNA index adapters were added via incubation for end repair. The cDNA fragments were amplified by PCR, purified by Ampure XP Beads, and then dissolved in EB solution. The double-stranded PCR products were heated to denature and circularized by the splint oligo sequence to obtain the final library. The single-strand circular DNA (ssCir DNA) was formatted as the final library. The final library was amplified with phi29 to make DNA nanoballs (DNBs), which had more than 300 copies of one molecule. DNBs were loaded into the patterned nanoarray, and paired-end 100-base reads were generated on the BGISEQ500 platform (BGI-Shenzhen, China).

RNA-Seq data analysis

The sequencing data were filtered with SOAPnuke (v1.5.2) [20] by (1) removing reads containing sequencing adapters; (2) removing reads with low-quality base (base quality ≤5) ratios more than 20%; (3) removing reads with unknown base ('N' base) ratios more than 5%. The clean reads were stored in FASTQ format. The clean reads were mapped to the reference *P. falciparum* 3D7 genome (assembly GCA_000002765) using HISAT2 (v2.0.4)[21]. Bowtie2 (v2.2.5) [22] was applied to align the clean reads to the reference coding gene set, and then, the gene expression levels were calculated by RSEM (v1.2.12)[23] and normalized to fragments per kilobase of transcript per million mapped reads (FPKM) values. Functional annotation of genes was achieved by mapping genes to the Gene Ontology (GO, http://www.geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) databases using BLAST software (V2.2.23). GO annotation was performed by Blast2GO (v 2.5.0) with NR annotations. DESeq2 (v1.4.5)[24] was used to detect differentially expressed genes (DEGs), and DEGs with fold change > 2 or < -2 and adjusted *P* value ≤ 0.001 were considered to be significant DEGs. GO enrichment analysis was performed using Phyper (https://en.wikipedia.org/wiki/Hypergeometric_distribution), a function of R. The significant levels of terms and pathways were corrected by Q value with a rigorous threshold (Q value ≤ 0.05).

Results

Successful establishment of HRP2-knockout Plasmodium falciparum

The pUF1-BSD-Cas9 plasmid was constructed from the pUF1-Cas9 plasmid. Then, the *P. falciparum* 3D7 strain was transfected with 50 μg of pL6cs-hDHFR-hrp2 (donor DNA) and 50 μg of pUF1-BSD-Cas9 via electroporation. To select the successfully transfected parasite, BSD and WR99210 were added to the culture medium one day after electroporation.

Approximately 20 days after electroporation, live parasites could be seen in the culture under selection with the two drugs described above. A portion of the live parasite population was collected for genomic DNA isolation, and PCR was performed to validate the modification of the *Pfhrp2* gene. In these PCRs, two primers were designed for the genomic DNA sequences beyond the left and right homologous arms (P1/P2, Fig. 2) to prevent contamination from the episomal plasmid template. PCR products were analysed by agarose electrophoresis and sequenced for confirmation.

Transgenic parasites that had been checked by PCR and DNA sequencing were further checked by Southern blotting analysis. Two experiments were performed to verify that the *Pfhrp2* gene was replaced by the drug resistance gene hDHFR. One experiment was performed to indicate that the wild-type strain 3D7 still possessed the *Pfhrp2* gene while the transgenic parasite did not (Fig. 3b). The other experiment was conducted to prove that the *Pfhrp2* gene was replaced by the hDHFR gene (Fig. 3c). Southern blotting analysis results showed that only the hDHFR gene was detected in the transgenic parasite. Therefore, the *Pfhrp2* gene was successfully knocked out from the *P. falciparum* 3D7 strain using the CRISPR/Cas9 system.

Transgenic parasites that had been checked by PCR, DNA sequencing, and Southern blotting analysis were further confirmed by Western blotting analysis. The original molecular weight of the protein was 32.41 kD (Fig. 3a). Western blotting results showed that the molecular weight of HRP2 was not the same as its theoretical molecular weight. The band corresponding to HRP2 appeared to be slightly larger than its theoretical molecular weight, which may be a result of post-translational modification.

Therefore, the CRISPR/Cas9 system was successfully used to knock out the Pfhrp2 gene.

Overview of the RNA-Seq data

A total of 36 samples were tested using the BGISEQ-500 platform, and at least 6.0 Gb of clean data from each sample were obtained from transcriptome sequencing and were available for further expression level analysis after quality control. Total clean reads from the RNA-Seq data were mapped uniquely to the 3D7 genome. More than 97% of the total clean reads had Phred-like quality scores at the Q20 level (Additional file 5: Table S4).

The average comparison rate of the sample genome was 57.39%, and the average comparison rate of the comparison gene set was 49.26%; the number of predicted new genes was 77 (Additional file 6: Table S5); the total number of detected expressed genes was 5,427, of which 5,350 were known genes; 77 new genes were predicted; and a total of 2,358 new transcripts were detected, of which 2,078 belonged to new alternative splicing subtypes of known protein-coding genes, and 77 belonged to new protein-coding gene transcripts. The remaining 203 transcripts were long non-coding RNAs. Length distribution of transcripts is listed in Additional file 7: Table S6. Via comparative transcriptome analysis, these RNA-Seq data provided a solid foundation for identifying the genes participating in biological processes.

Analyses of differentially expressed genes

To investigate changes in gene expression profiles, DESeq2 was used to detect DEGs, and DEGs with fold change > 2 or < -2 and adjusted *P* value $\leq$ 0.001 were considered to be significant DEGs. Differential expression analysis identified 964, 1261, 3138, 1064, 2512 and 1778 DEGs in the comparison groups 3D7_0h vs. L2_0h, 3D7_8h vs. L2_8h, 3D7_16h vs. L2_16h, 3D7_24h vs. L2_24h, 3D7_32h vs. L2_32h, and 3D7_40h vs. L2_40h, respectively. Differential expression analysis for the comparison groups 3D7_0h vs.

L2_0h, 3D7_8h vs. L2_8h, 3D7_16h vs. L2_16h, 3D7_24h vs. L2_24h, 3D7_32h vs. L2_32h, and 3D7_40h vs. L2_40h identified 373, 520, 1499, 353, 1253, and 742 upregulated genes, respectively, and 591, 741, 1639, 711, 1259, and 1036 downregulated genes, respectively (Fig. 4). The DEGs identified in the comparison groups can help us understand the gene regulatory mechanisms underlying the parasite response to knockout of the Pfhrp2 gene.

A total of 5 DEGs related to haem metabolism and synthesis were identified (Additional file 8: Table S7), including genes encoding delta-aminolevulinic acid synthetase (ALAS, PlasmoDB:PF3D7_1246100), ferrochelatase (FC, PlasmoDB:PF3D7_1364900), haem oxygenase (HO, PlasmoDB:PF3D7_1011900), stromal-processing peptidase (SPP, PlasmoDB:PF3D7_1440200), and porphobilinogen deaminase (PBGD, PlasmoDB:PF3D7_1209600) (Fig. 5). The ALAS and FC genes were found to be significantly upregulated in the comparison groups. The HO, SPP, and PBGD genes were found to be significantly downregulated in the comparison groups.

Enrichment analysis of GO terms

Functional annotation and classification were performed by comparing the sequences with the GO database. Enrichment analyses of GO terms were performed for DEGs using the phyper function in R software. The P values were calculated, and then, FDR correction was performed on the P values. Usually, a function with a Q value ≤ 0.05 is regarded as being significantly enriched. As PfHRP2 mediates haemozoin formation and is involved in haem-related processes, DEGs in the comparison groups were primarily associated with GO terms related to haem metabolism processes, HO (decyclization) activity, and haem biosynthetic processes. However, no GO terms were significantly enriched in the abovementioned haem-related process (Q value =1).

Discussion

The traditional method to edit *P. falciparum* genes is very inefficient and requires several months for knock-in/out of target genes. This greatly limits molecular studies on malaria parasites. Recently, CRISPR/Cas9 has been used for gene editing in various organisms, including *Plasmodium* species[15, 16, 25].

Our CRISPR/Cas9 system contains homologous arms (donor DNA fragments), sgRNA, and a selectable marker in one plasmid and Cas9 nuclease with a selectable marker in another plasmid. Twenty days after electroporation, specific gene-disrupted parasites appeared. In this study, we successfully applied this CRISPR/Cas9-based genome editing system to disrupt the *Pfhrp2* gene of *P. falciparum* 3D7.

We observed that the growth of the parasite in the host was not affected by the deletion of *Pfhrp2* compared with the growth of the wild type. Furthermore, HRP2 gene deletion mutants of *P. falciparum* of multiple genetic origins were previously found in South America, Asia, and Africa[26-29]. These results all proved that *Pfhrp2* is not an essential gene for the survival of parasites during intraerythrocytic stages.

Malaria parasites have haemoglobin-derived haem metabolism and *de novo* haem biosynthesis pathways. During the intraerythrocytic stages, parasites ingest host cell haemoglobin within the food vacuole to supply amino acids for growth and release toxic haem. The released by-product haem can bind with HRPII and HRP III to become haemozoin [12]. HO may enzymatically degrade some haem to biliverdin (BV) or its downstream metabolite bilirubin (BR) [30].

According to the RNA-Seq data, with disruption of *Pfhrp2,* the transcript level of *PfHO* was significantly downregulated, which further affected haem metabolism. At the same time, the transcripts levels of genes encoding enzymes related to the *de novo* haem biosynthesis pathway in the mitochondrion of *P. falciparum* 3D7, such as ALAS (the first enzyme of the pathway) and FC, were upregulated for increased haem supply for the parasite. However, the transcript expression of PBGD, which catalyses the conversion of porphobilinogen to hydroxymethylbilane in the apicoplast of the parasite, was downregulated. This may decrease haem biosynthesis in the apicoplast of the parasite.

P. falciparum contains a vestigial, non-photosynthetic plastid, the apicoplast. Numerous proteins encoded by nuclear genes are targeted to the apicoplast because of N-terminal extensions. The first part of this leader sequence is a signal peptide that targets proteins to the secretory pathway. The second so-called transit peptide region is required to direct proteins from the secretory pathway across the multiple membranes surrounding the apicoplast. The transit peptide of apicoplast-targeted proteins are cleaved and then imported into the apicoplast. SPP performs this cleavage reaction. SPP also shares a leader sequence with Δ-aminolevulinic acid dehydratase (ALAD) via an alternative splicing event [31-33]. ALAD is also involved in the haem biosynthesis pathway. The SPP gene was significantly downregulated in the comparison groups. This would decrease the amount of protein imported into the apicoplast and finally affect haem biosynthesis in the apicoplast.

When *Pfhrp2* was disrupted, haem metabolism in the food vacuole and haem biosynthesis in the apicoplast were influenced, and the gene transcript levels of the enzymes participating in haem biosynthesis in the mitochondrion of the parasite were upregulated. It was reported that vestigial host enzymes within the erythrocyte cytoplasm can replace the haem biosynthesis process in the apicoplast[34]. For this reason, *de novo* haem biosynthesis can reduce the influence of the downregulation of PBGD and SPP. Combined with the observation that the growth of parasites did not change, the results indicate that the haem level in the parasite remained stable. LC-MS/MS and metabolomics investigation should be conducted to confirm this in the near future.

It was reported that the haem biosynthesis pathway is nonessential in the blood stage because ALAS and FC gene knockouts did not affect parasite survival. This conclusion is inappropriate because parasites can acquire haem from two pathways for survival in the blood stage. When one pathway encounters a problem, the other pathway provides haem for the parasite. Our research proved that haem metabolism and the haem biosynthesis pathway are both needed at the gene transcript level in the blood stage for parasite survival.

Chloroquine was discovered and derived from quinine in 1934[35]. It is effective against the malarial parasite during its intraerythrocytic stages. It can inhibit the HRP-mediated synthesis of haemozoin and disrupt the haem pathway that occurs within the acidic digestive vacuole of the parasite [12]. Our research has shown that intraerythrocytic-stage parasites not only possess a haem biosynthesis pathway but also can use host enzymes and substances to assist in haem biosynthesis. The FC knockout parasite cells grew normally and exhibited no changes in sensitivity to haem-related antimalarial drugs[36]. Parasites can acquire haem from two different pathways, and disruption of only *Pfhrp2* or enzymes of haem biosynthesis cannot exterminate the haem supplement completely during intraerythrocytic stages. Therefore, haem metabolism and the haem biosynthesis pathway are not viable targets for traditional drug inhibition[13, 36]. A cooperative mechanism exists between the haem biosynthesis and metabolism pathways for parasite growth and survival in the blood stage. It may be possible to disrupt haem supplementation by simultaneous suppression of haem metabolism and the haem biosynthesis pathway. We believe that our work will be beneficial for understanding haem acquisition and drug resistance during intraerythrocytic stages.

Conclusion

The results suggested that disruption of *Pfhrp2* alters the parasite's haem metabolic and biosynthesis pathways at the gene transcript level. A cooperative mechanism exists between the haem biosynthesis and metabolic pathways for parasite growth and survival in the blood stage. It is difficult to treat malaria patients by inhibiting only one pathway with traditional antimalarial drugs.

Abbreviations

BSD: blasticidin S deaminase; sgRNA: single-guide RNA; hDHFR: human dihydrofolate reductase; DSB: double-strand break;

Declarations

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analysed during the current study are available under NCBI project PRJNA663197 (https://www.ncbi.nlm.nih.gov/bioproject/663197) with accession numbers for 72 objects (SAMN16122358~SAMN16122429). Any reasonable requests should be made to the corresponding author.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YY and QC designed the research. YY, TT, BF, SL, and NH performed the research. XM, XX, and LJ analysed the data. YY wrote the paper. All authors read and approved the final manuscript.

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Figures

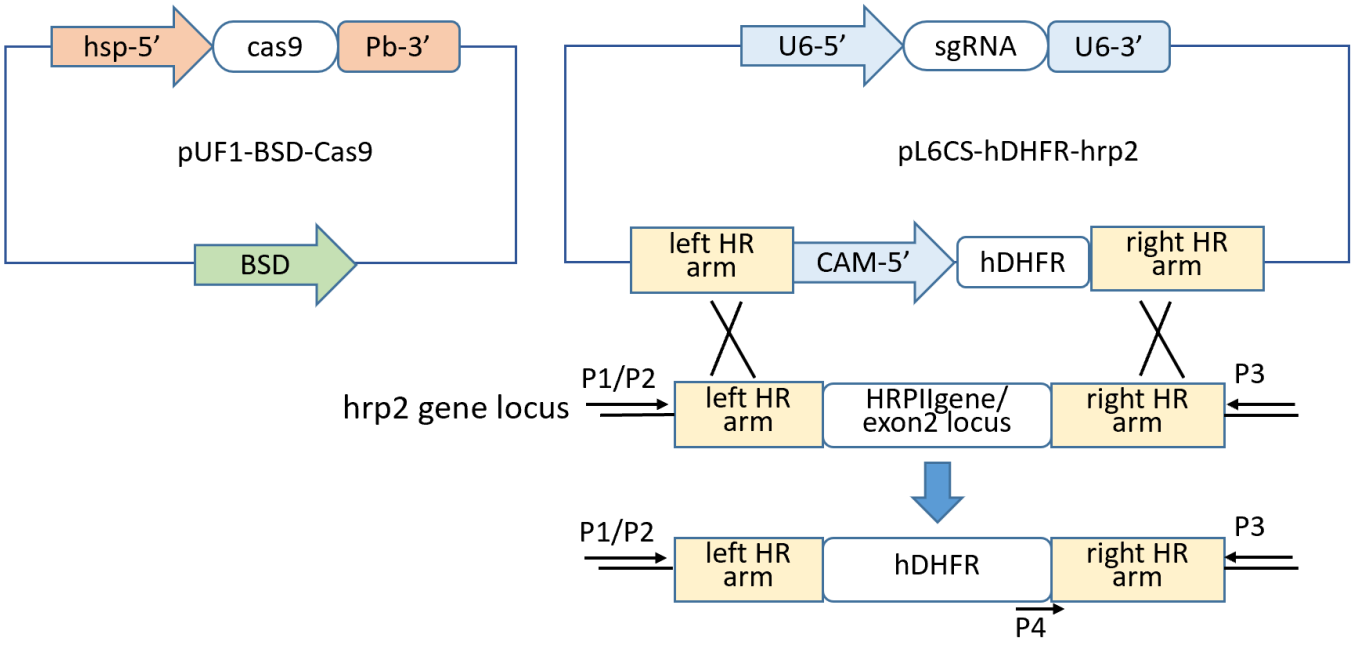

Figure 1

Schematic illustration of the underlying principle of hrp2 gene deletion using CRISPR/Cas9. The hrp2 gene was replaced by hDHFR sequences through homologous recombination that occurred at the left and right arms. Primers for PCR to check for hDHFR are labelled P1 and P2.

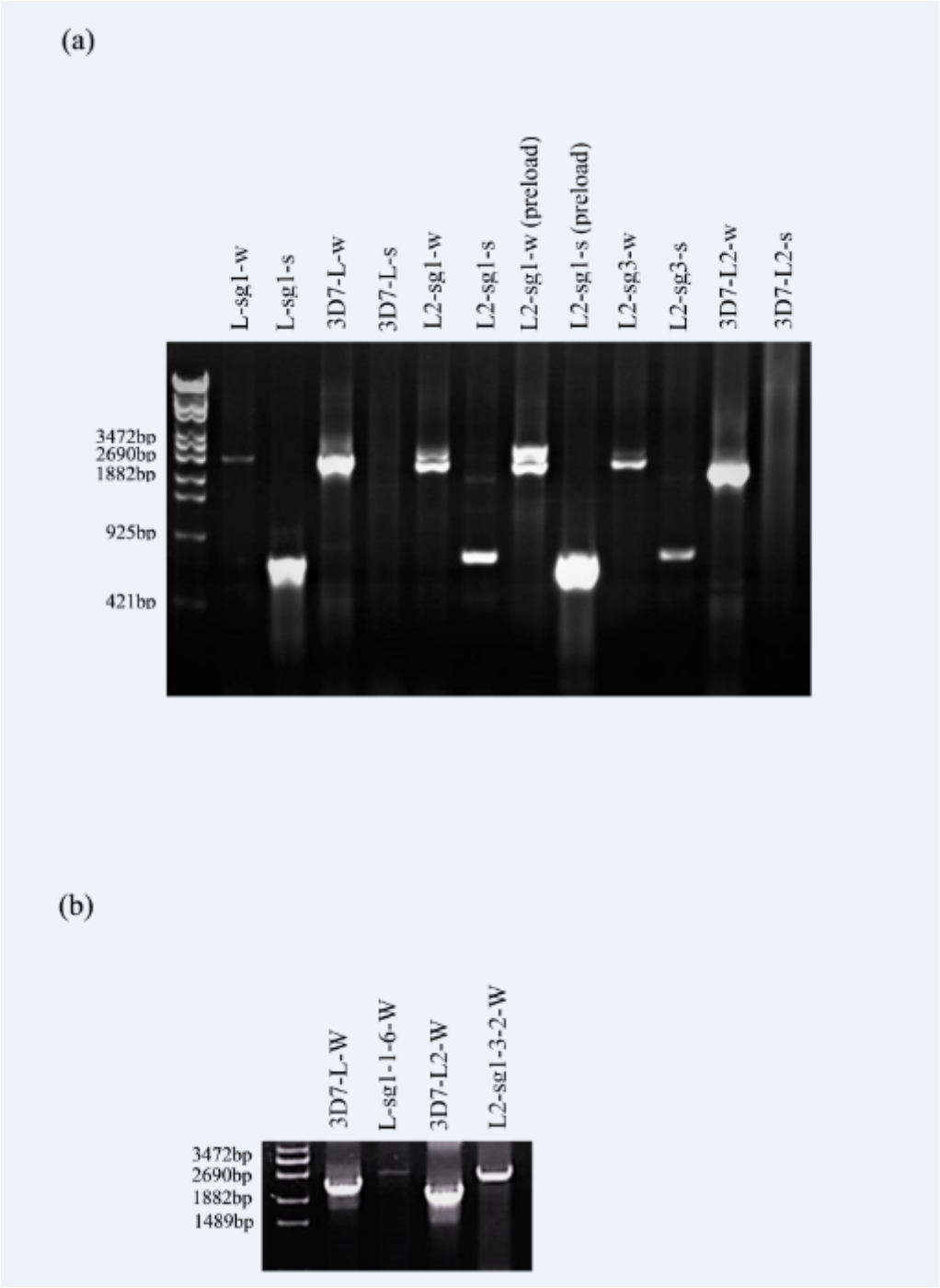

Figure 2

Genomic DNA PCR to confirm gene disruption. (a) Genomic DNA PCR to confirm the successful modification of the Pfhrp2 gene by the sgRNA. (b) Genomic DNA PCR to verify the hrp2- monoclonal parasites. 3D7 was the wild type of Plasmodium falciparum. L and L2 were the transgenic parasites that were disrupted in exon 1 plus exon 2 and only exon 2 of Pfhrp2, respectively. The PCR product sizes of the whole fragment (w) and partial fragment (s) were 2903 bp and 725 bp, respectively.

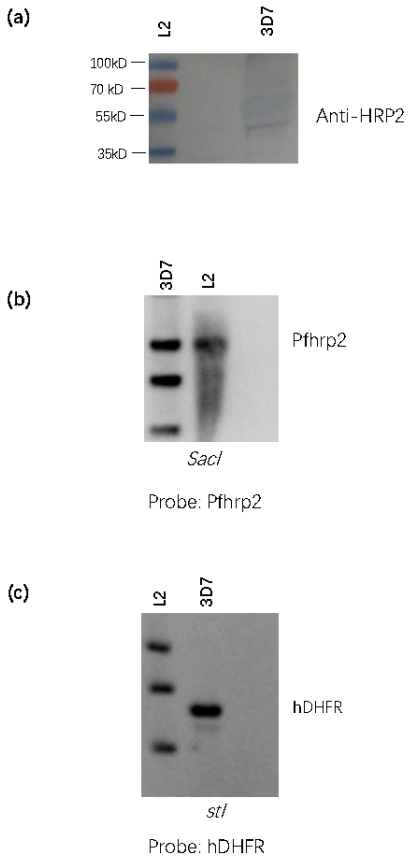

Figure 3

(a) Western blotting analysis to confirm the expression of the PfHRP2 protein. The supernatants of the parasite culture medium were separated by SDS-PAGE, and a mouse monoclonal [MFPG-55P] antibody against Plasmodium falciparum (HRP) was the primary antibody used for the Western blotting analysis to confirm HRP2 protein expression. HRP-goat anti-mouse was the secondary antibody. (b) Southern blotting analysis to confirm the disruption of Pfhrp2. The genomic DNA was digested overnight using the

SacI restriction endonuclease. The DNAs were separated on an agarose gel and transferred to a membrane. The blots were hybridized with the labelled Pfhrp2 probe and exposed for 10 mins. (c) Southern blotting analysis to confirm the presence of the drug resistance gene hDHFR. The genomic DNA was digested overnight using the PstI restriction endonuclease. The DNAs were separated on an agarose gel and transferred to a membrane. The blots were hybridized with the labelled hDHFR probe and exposed for 40 min. L2 was the transgenic parasite, which was disrupted in only exon 2 of Pfhrp2.

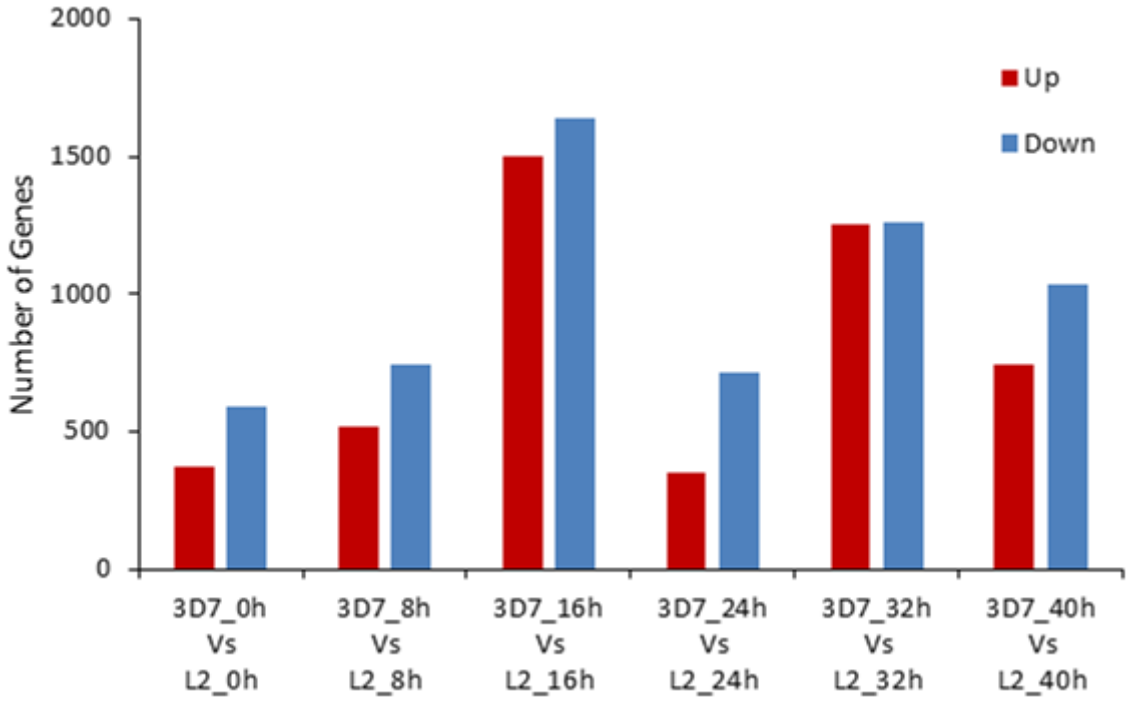

Figure 4

Statistics of differentially expressed genes and gene regulation in the comparison groups.

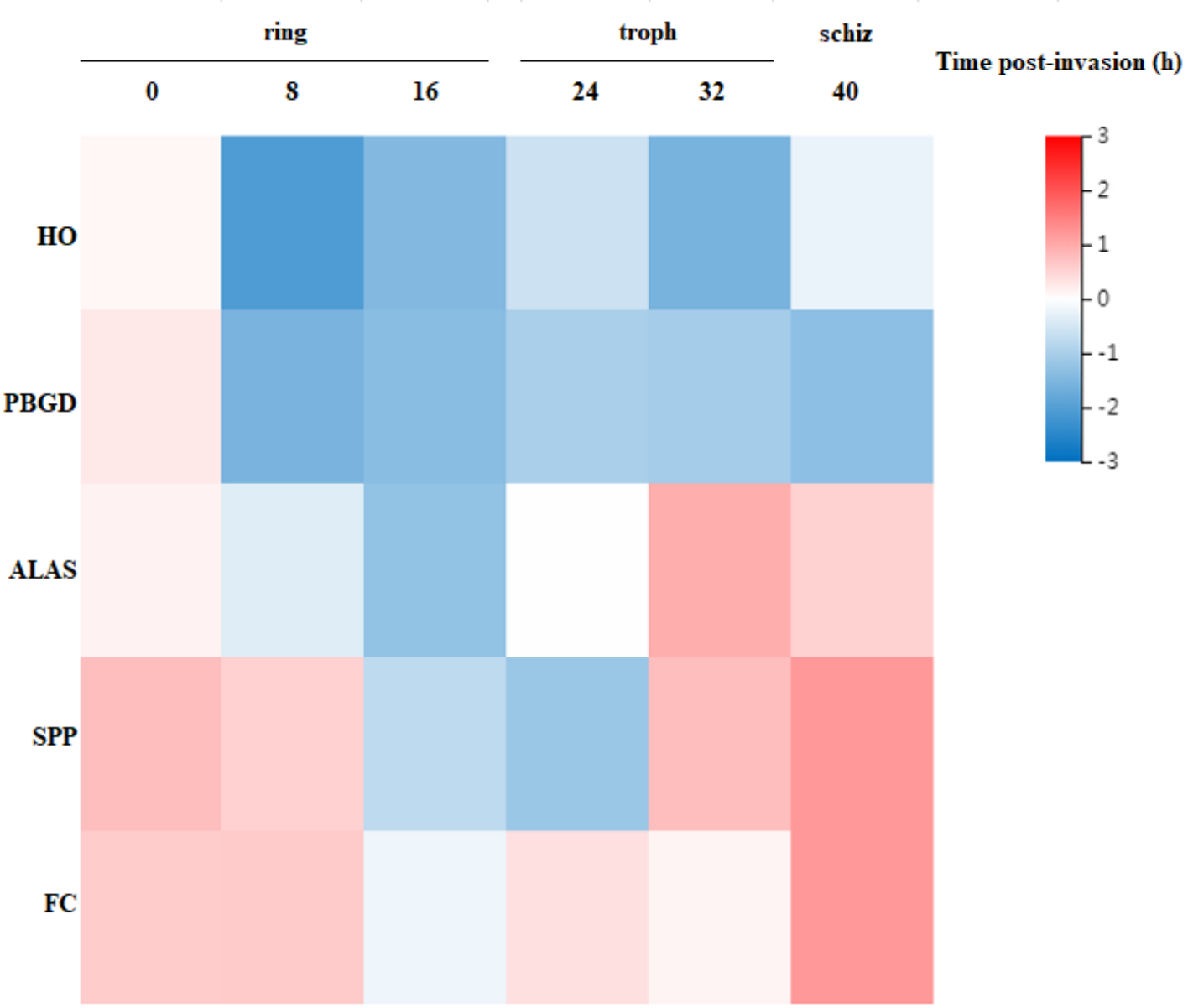

Figure 5

Transcription pattern of the genes involved in haem metabolism. Heat map representation of the relative transcription activity of 5 genes at six points during asexual erythrocytic growth. The horizontal axis represents the expression level of each gene calculated as Log2(FPKM+1). The values range from -3 (lowest, blue) to +3 (highest, red). HO (haem oxygenase, PlasmoDB:PF3D7_1011900), PBGD(porphobilinogen deaminase, PlasmoDB:PF3D7_1209600). ALAs (delta-aminolevulinic acid synthetase, PlasmoDB:PF3D7_1246100) , SPP(stromal-processing peptidase, PlasmoDB:PF3D7_1440200), FC (ferrochelatase, PlasmoDB:PF3D7_1364900). .

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