

Expression of *Plasmodium falciparum* genes involved in erythrocyte invasion varies among isolates cultured directly from patients

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Abstract

Plasmodium falciparum merozoites invade erythrocytes using a range of alternative ligands that includes erythrocyte binding antigenic proteins (EBAs) and reticulocyte binding protein homologues (Rh). Variation in the expression of some of these genes among culture-adapted parasite lines correlates with the use of different erythrocyte receptors. Here, expression profiles of four *Rh* genes and *eba175* are analysed in a sample of 42 isolates cultured from malaria patients in Kenya. The profiles cluster into distinct groups, largely because of very strong negative correlations between the levels of expression of particular gene pairs (*Rh1* versus *Rh2b*, *eba175* versus *Rh2b*, and *eba175* versus *Rh4*), previously associated with alternative invasion pathways in culture-adapted parasite lines. High levels of *eba175* are seen in isolates in expression profile group I, and may be associated with sialic acid-dependent invasion. Groups II and III are, respectively, characterized by high levels of *Rh2b* and *Rh4*, and are more likely to be associated with sialic acid-independent invasion.

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1. Introduction

Analyses of culture-adapted lines of *Plasmodium falciparum* have revealed several different receptor–ligand interactions mediating alternative pathways of erythrocyte invasion by merozoites [1,2]. The importance of several erythrocyte binding antigens (EBA) and reticulocyte binding protein homologues (Rh) has been demonstrated by parasite gene knock out studies and invasion assays using erythrocytes treated with enzymes that cleave different receptors [3–9]. In the development of blood stage parasites, gene transcript and protein synthesis of these molecules is highly specific to late stage schizonts [10,11].

There are differences among laboratory lines of *P. falciparum* in the levels of expression of Rh proteins [6,9,12], and some of these appear to be associated with use of different erythrocyte receptors in merozoite invasion. For example, parasite lines that express high levels of Rh1 tend to express low or undetectable levels of Rh2a and Rh2b and have a sialic acid-dependent mechanism of invasion, whereas parasites expressing less Rh1 are more likely to express Rh2a and Rh2b and undergo a sialic acid-independent invasion [6,9].

Although the invasion phenotype appears to be a stable property of most parasite clonal lines in culture, switching of invasion phenotype has been shown to occur in the Dd2/W2mef clone [13] and more recently in the CSL2 clone [8]. In these lines, a switch of invasion phenotype from sialic acid dependent to independent can be selected by culturing in neuraminidase-treated erythrocytes, and involves increased transcription of *Rh4* [8,14]. Disruption of the *eba175* gene by transfection also causes a switch to sialic acid independent invasion and upregulation of

Abbreviations: EBA, erythrocyte binding antigenic protein; Rh, reticulocyte binding protein-like homologue; RT, reverse transcription

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Rh4 transcription [8]. However, a comparison of four parasite lines that do not undergo switching indicated that variation in the level of *Rh4* gene transcription was unrelated to the sialic acid dependence of the lines [14].

Field isolates of *P. falciparum* have a range of phenotypes similar to that of diverse culture-adapted lines [15–17], but gene expression patterns have not been investigated. As selection among parasites occurs during growth in laboratory culture, and some clones can switch their expression profile, it is important to know the nature of variation among parasites in humans. As a survey of the variation in gene expression that occurs within a single *P. falciparum* endemic population, the *ex vivo* generation of parasites in 42 blood samples from malaria patients in Kenya were grown to schizont stage in short term culture, and relative levels of transcripts of the different protein coding *Rh* genes and *eba175* were assayed. The transcript profiles clustered into distinct groups, and there were strong negative correlations among the isolates in the expression of particular pairs of genes. There were no detected differences in genomic copy numbers of any of the genes, indicating that the profiles are under transcriptional control. A subsequent preliminary analysis of two other protein coding *eba* genes in a subset of the isolates indicated less variation in relative transcript levels than for *eba175*.

2. Materials and methods

2.1. Blood samples and parasite culture

A total of 42 blood samples from children presenting with *P. falciparum* infections of >1% parasitaemia at Kilifi District Hospital were cultured for between 24 and 36 h to schizont stage of development. Sixteen of these were cultured fresh upon collection, from patients that presented during two consecutive rainy seasons (5 during December 2003–January 2004, and 11 during June–August 2004), and 26 had been cryopreserved upon collection several years previously (from 1994 to 1996) and were thawed for culture (during June–August 2004). Cases of severe malaria that were admitted to the pediatric ward with unrousable coma (Blantyre score ≤ 2), prostration (inability to sit or to breast feed) or respiratory distress (abnormally deep breathing) accounted for 22 of the isolates (12 in 1994–1996, 10 in 2003–2004). The other 20 isolates were from patients with uncomplicated mild clinical malaria attending the Outpatients Department (14 in 1994–1996, 6 in 2003–2004).

Erythrocytes in heparinised blood from the patients were separated from lymphocytes and buffy coat by LymphoprepTM density gradient centrifugation, washed and cultured at 2.5% haematocrit in RPMI complete medium until schizont stage of development in 3% CO₂, 1% O₂, and 96% N₂ at 37 °C. Packed volume varied between 50 and 300 μ l depending on availability of volumes within samples being shared with other studies. For DNA analysis, approximately 200 μ l of each parasite culture (equivalent to 4 μ l of packed erythrocytes) was sampled before growth, and DNA extracted using the QIAampTM DNA Blood Mini Kit (Qiagen, UK). From each of the 26 isolates that were cultured after cryopreservation, the schizont stage cul-

ture was centrifuged and cells (resuspended at 50% haematocrit in incomplete medium) harvested directly for parasite RNA. Approximately four volumes of TRI ReagentTM (Sigma) or TRIzol ReagentTM (Invitrogen) was added to the erythrocytes prior to freezing at -80 °C. The parasites from the 16 fresh isolates were enriched at schizont stage using 55% Percoll, then diluted to 1% parasitaemia with heterologous erythrocytes for invasion assays, at which point part of the sample was frozen as above.

2.2. Erythrocyte invasion assays

Erythrocytes from an uninfected Caucasian donor (blood group O+) were treated with 1 mg/ml trypsin, 1 mg/ml chymotrypsin or 50 mU neuraminidase for 1 h at 37 °C, after which they were washed once with 1 ml incomplete RPMI and then 0.5 mg/ml soybean trypsin inhibitor was added to the trypsin and chymotrypsin samples to inhibit the enzyme (incomplete RPMI was added to the control and neuraminidase sample) while mixing on a rotating wheel for 10 min at room temperature. Erythrocytes were washed three times before resuspending at 50% haematocrit in RPMI, and stored at 4 °C for a maximum of 1 week, prior to use in invasion assays. Cultured schizonts from the fresh isolates were enriched by centrifugation through 55% Percoll, washed twice and adjusted to around 1% parasitaemia with untreated or enzyme-treated donor erythrocytes. These comprised a subset of the isolates tested in a larger study of parasite growth and invasion phenotypes ([18]; Deans et al., in preparation). They were cultured for a further 24 h in duplicate wells of 96-well plates in volumes of 50 μ l at 2% haematocrit, following which thin blood films of the pre-invasion sample and the ring-invaded erythrocytes were prepared and stained with Giemsa for determination of parasitaemia. At least 1000 erythrocytes were counted from each the wells. Parasite multiplication rate (PMR) was scored as the number of ring-infected erythrocytes after invasion divided by the pre-invasion parasitaemia. Percentage inhibition of invasion due to enzyme treatment of erythrocytes was measured as $100 - ((\text{enzyme-treated PMR}/\text{control PMR}) \times 100)$.

2.3. RNA extraction and reverse transcription

RNA was extracted from the preparation from each isolate in TRIzolTM or TRI ReagentTM according to manufacturer's instructions, in combination with the RNeasy MicroTM (Qiagen, UK) protocol for removal of DNA contamination. The extracted RNA was frozen in two 5 μ l aliquots and a third aliquot (2 μ l) was used for estimation of the RNA concentration using the Agilent RNA labchip. Total RNA was reverse-transcribed with random primers using TaqMan reverse transcription reagents (Applied Biosystems, UK) in a 50 μ l reaction, for 10 min at 25 °C, followed by 1 h at 37 °C. Calculations were made so that for each reverse transcription (RT) reaction no more than 800 ng of RNA was used, and this was split between eight PCR assays so the cDNA used in each PCR reaction would not exceed 100 ng (the RNA yield varied so that this was frequently less than 50 ng and could be as low as 1 ng per PCR reaction).

Table 1
Sequences of primers and probes for each of the genes analysed by real time PCR*

Gene	Sequence	Nucleotide position	Accession no.
<i>Rh1</i> cDNA (exon–exon)	F: 5'-GCAAAGGTGGATTTTCTGCAACAT-3'	3–26	AF533700
	R: 5'-TCTGAATAGTCCTTTCTTATTTTTTTCATTGGAAGA-3'	RC: 82–116	
	P: 5'-FAM-CATGGCTAAATTCTGCTAAGTAA-MGB-3'	RC: 45–67	
<i>Rh1</i> gDNA	F: 5'-GGGAAAATATTAATAATGTGGAAATATGGTATGAAAAAATATAATTG-3'	4307–4354	AF533700
	R: 5'-ATGTATCAATGTTTTCCATATATTTGCAGCTTT-3'	RC: 4390–4423	
	P: 5'-FAM-CATTCATATGACGTAAGAAATAT-MGB-3'	RC: 4356–4378	
<i>Rh2a</i>	F: 5'-GGTCATAAAAGATAATGAGACACAATTGAAAACAA-3'	8805–8839	AY138496
	R: 5'-GCAATATTTTTTCTGGTTTTCTTCGTATTCC-3'	RC: 8907–8938	
	P: 5'-FAM-CACGTTTCAAATTAAGATATAATTG-MGB-3'	RC: 8863–8887	
<i>Rh2b</i>	F: 5'-ACAGAAAGCGATGATATTGATAACAGTGAA-3'	8521–8550	AY138500
	R: 5'-CCCATGGGTGTTACTTCTATGACT-3'	RC: 8596–8619	
	P: 5'-FAM-CATATGAGTGACATCGAAAGTA-MGB-3'	8566–8587	
<i>Rh4</i>	F: 5'-GAAATGACGCAATCCCTCAAAGA-3'	68–92	AF432854
	R: 5'-GGTGTGTTTTATTTATATCATGTTGATCTGTGA-3'	RC: 127–160	
	P: 5'-FAM-CTTCTGGATCGTTTTTTTTT-MGB-3'	RC: 93–112	
<i>ama1</i>	F: 5'-GGATTATGGGTCGATGGAAATTGTG-3'	937–961	U65407
	R: 5'-CATAATCTGTTAAATGTTGTTTCATATTGTTTAGGTTGAT-3'	1044–1081	
	P: 5'-FAM-CCGAAGCACTCAATTCA-MGB-3'	RC: 1026–1042	

Nucleotide position corresponds to the first nucleotide of the forward primer in the 3D7 coding sequence of each gene. RC indicates the reverse complement sequence that is aligned with the full length coding sequence.

* *eba* genes were analysed using primers and probes as previously described [11].

2.4. Real time quantitative PCR analysis of parasite cDNA

For relative quantification of different gene transcripts the cDNA was used in a fluorogenic 5'-nuclease assay using the TaqMan system on the ABI Prism 7000 Sequence Detector (Applied Biosystems). Gene-specific TaqMan primers and probe sets were obtained using the Assays-by-Design system (Applied Biosystems) for the *Rh1*, *Rh2a*, *Rh2b*, *Rh4* and *ama1* genes (Table 1). For *eba175*, *eba140* and *eba181*, primers and probes were based on those previously described [11]. The chosen sequences for amplification were not designed to span an exon–exon boundary, except in the case of *Rh1*. All probes were labeled with 6-carboxy-fluorescein (FAM) on the 5'-end and a non-fluorescent quencher (MGB-NFQ, Applied Biosystems) on the 3'-end and used in single reporter assays. The PCR reactions were carried out in 25 μ l volumes using 900 nM of each primer and 250 nM probe.

Serial dilutions of Dd2 genomic DNA (four dilutions over a 1000-fold concentration range) were used to generate standard curves for each gene individually (except for *Rh1* as this gene has multiple copies in the Dd2 genome [9] and the *Rh1* oligonucleotides were designed to detect cDNA rather than genomic DNA). As the efficiency (derived from the slope of the standard curve) for all the gene assays was comparable, the amount of *Rh1* transcript was estimated using the standard curve obtained for *Rh2a* within each plate reaction. Each 96-well plate run included the genomic DNA standard curves, cDNA from five field samples to be tested for each of the genes, and control wells to check for genomic DNA contamination (samples from each isolate without RT were tested for two different genes so the plate contained negative controls for all genes). Quantitation of each

gene in each sample was performed using the ABI Prism 7000 Sequence Detection System Software version 1.2.3. Genomic DNA standard curves were generated each time a set of samples was run, by plotting the C_t value for each standard well against the known dilutions. On all plates the threshold fluorescence value of 0.2 was chosen for C_t determination (after ensuring that this corresponded to the exponential part of the PCR amplification in each plate).

2.5. Statistical and cladistic analysis of gene expression profiles

For each isolate the transcript levels of each of the *Rh* genes and *eba175* were normalized against the transcript level of *ama1*, which has a similar stage-specific expression and is considered to be expressed at similar levels among different parasite lines. Expression profiles were generated by plotting the relative proportions of each transcript, calculated as a percentage of the total for the five genes.

Spearman's rank nonparametric correlation coefficient (ρ) was used to measure the pairwise relationships between the relative proportions of expression of each of the different genes across all isolates. Mann–Whitney *U* tests were used to assess whether there were significant associations between the relative amounts of each of the transcripts and the case status of the patients (severe or mild malaria) and whether or not the isolate was freshly cultured or had been cryopreserved. Spearman's ρ was also used to test for correlation between relative levels of expression and parasitemia or multiplication rate. All statistical analysis was performed using SPSS version 11.0 software.

The expression profiles of the five candidate genes together were compared across different isolates by computing a difference index for each pair of isolates. For each gene, the difference in relative proportions of different transcripts between the two isolates was divided by the sum of the proportions in the two isolates, and this value was summed over all five genes and the total divided by five (to give a standardized difference index with a potential range from 0 to 1.0). The resulting distance matrix among all isolates was then analysed using neighbour-joining and UPGMA clustering analysis with the phylogenetic software PHYLIP (PHYLogeny Inference Package available free at <http://evolution.genetics.washington.edu/phylip.html>). An additional analysis was performed using NTSys (Numerical Taxonomical Systems, Exeter Software) on the relative expression levels for each of the five genes, generating an independent distance matrix and UPGMA tree to check the integrity of the major clusters derived by the above method.

2.6. Testing for multiple clone infections and genomic amplification of gene copy numbers

To test for the presence of multiple clones of *P. falciparum* in the isolates, the highly polymorphic repeat sequences in two loci (*msp1* block 2 and *msp2* block 3) were amplified using a nested PCR method with allele type-specific internal primers [19].

For estimation of genomic copy numbers of each of the candidate genes, genomic DNA was analysed in the TaqMan system, following a method similar to that used for analysis human gene copy number variation [20]. The gene-specific primers and probe sets were the same as for the transcript analyses, except for *Rh1* for which a different set of primers and probes was used for genomic DNA that did not span a cDNA exon–exon boundary (Table 1). Every assay included standard curves for each gene of interest (four dilutions of 3D7 genomic DNA covering 1000-fold differences) and was replicated with two different standard single copy genes (*ama1* on one assay and *seryl tRNA synthase* on another assay). Non-template wells without DNA served as negative controls, and separate wells with 3D7 (single genomic copy of *Rh1*) and FCB1 (multiple genomic copies of *Rh1*) DNA as positive controls.

3. Results

3.1. *P. falciparum* field isolates vary in the relative expression of *Rh* genes and *eba175*

The gene expression profile was determined for a total of 42 isolates cultured to schizont stage *ex vivo* (16 fresh and 26 thawed cryopreserved samples). The levels of *Rh1*, *Rh2a*, *Rh2b*, *Rh4* and *eba175* transcripts were normalized against *ama1*. The *ama1* gene was chosen as it has similar stage specificity (being expressed in schizonts and merozoites) and is an essential gene that is not considered to vary in transcription among different parasite lines, so it should normalize for differences in the maturity of the schizonts as well as variation in parasitaemia among different isolates. Expression profiles were generated to analyse the relative amounts of each of the five transcripts (*Rh* genes

and *eba175*) considered to potentially determine invasion phenotypes. Visual examination of the plotted expression profiles indicates that isolates vary substantially in the relative levels of transcripts of the different genes (Fig. 1A and Supplementary figure). For example, in many isolates *eba175* transcript was abundant, whereas in some others it was relatively low and *Rh2b* was predominant. In almost all isolates the level of *Rh4* expression was substantially lower than the other transcripts, but in two isolates it was a predominant transcript. The variance/mean ratio of relative transcript abundance over all 42 isolates was highest for *Rh4* with a value of 8.76, reflecting the fact that it is very low in most isolates and abundant in only a few. For the other transcripts, the variance/mean ratio was approximately half of this or less, with values of 2.99, 3.42, and 4.77 for *Rh1*, *Rh2a* and *Rh2b*, respectively, and 4.58 for *eba175*.

Categorization of expression profiles into discrete groups was supported by a cladistic analysis. The analysis allowed the division of the 42 samples into three major groups (Fig. 1B). The first group (clade I) accounts for most (81%, 34/42) of the isolates and is characterized by a relative abundance of *eba175* transcript in most cases, with varying levels of the other transcripts (Fig. 1A and Supplementary figure). It may putatively contain more homogeneous subgroups, such as Ia and Ib that differ in the degree to which *eba175* expression is dominant and together account for 62% of all isolates (26/42, Fig. 1A and B). Members of subgroup Ia also express relatively high levels of *Rh1*, *Rh2a* or *Rh2b* whereas subgroup Ib is characterized by a greater predominance of *eba175* relative to the second most abundant transcript (either *Rh1* or *Rh2b*). The remaining eight isolates in group I did not fall into subgroup Ia or Ib (Fig. 1B and Supplementary figure). Group II consists of six isolates (14% of the total) expressing *Rh2b* as the predominant transcript, while group III consists of two isolates (5% of the total) that express *Rh4* as the predominant transcript (Fig. 1A and B). The same three major cladistic groups were obtained by neighbour-joining clustering of the expression distance matrix, and by an UPGMA analysis of distances independently derived using NTSys (trees not shown).

3.2. Strong negative correlations between expression of particular transcripts

Relationships between the relative proportions of different transcripts were explored for all ten pairwise correlations among the five candidate genes, from which three highly significant correlations emerged (Fig. 2). Two of these involved *eba175*, with very strong negative correlations between levels of *eba175* and *Rh2b* (Spearman's $\rho = -0.717$, $P < 0.0001$), and between levels of *eba175* and *Rh4* ($\rho = -0.526$, $P < 0.0001$). There was also a strong negative correlation between expression levels of *Rh1* and *Rh2b* ($\rho = -0.479$, $P < 0.001$). There were two weaker correlations, *Rh2a* and *Rh4* levels being negatively correlated ($\rho = -0.344$, $P < 0.025$), and *Rh2a* and *Rh2b* levels being positively correlated ($\rho = 0.324$, $P < 0.037$). The remaining five pairwise correlations were not significant (ρ values ranged from -0.275 to 0.286).

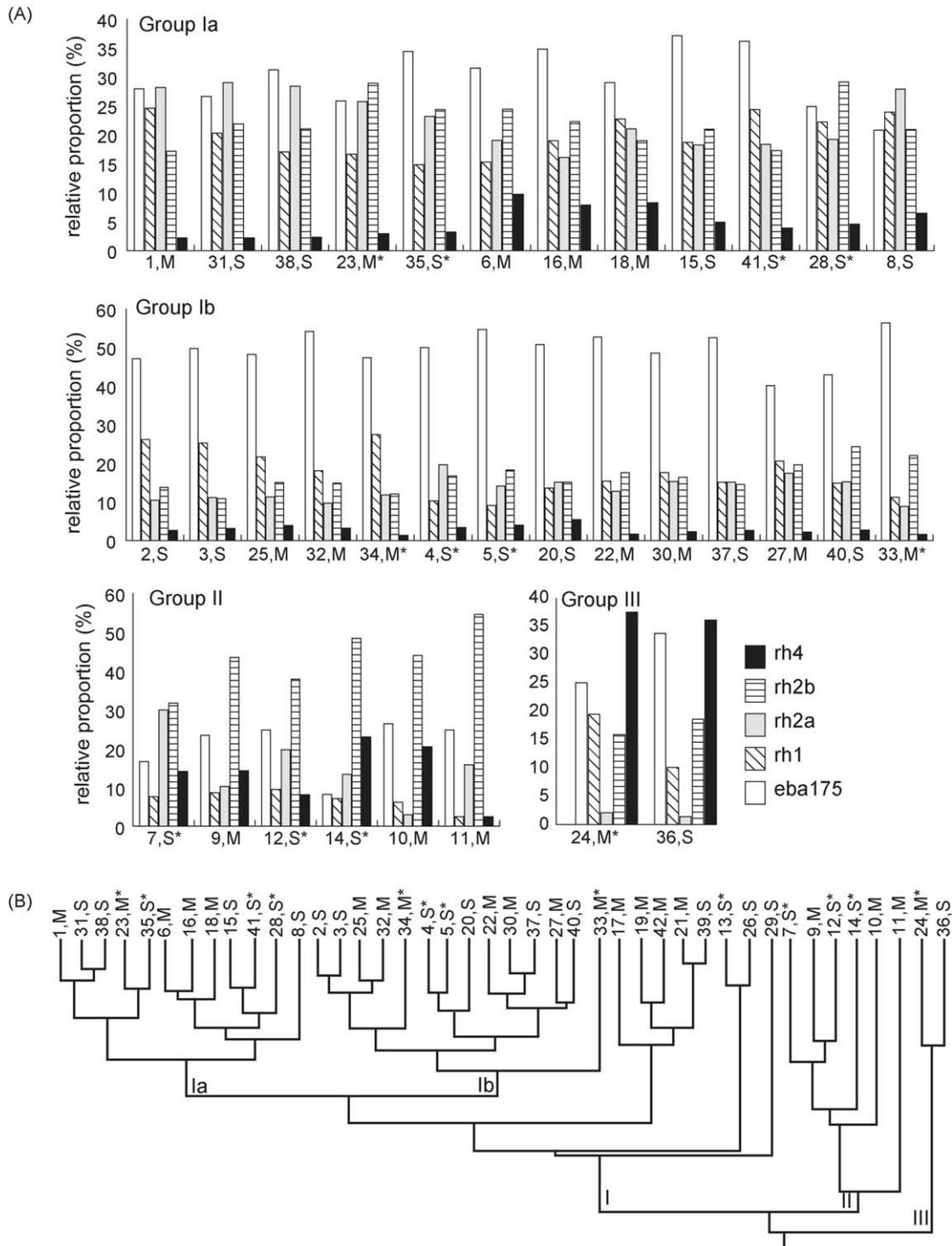


Fig. 1. Field isolates vary in the relative expression of *Rh* genes and *eba175* with discrete expression clusters apparent. (A) Histograms show the relative measured values of each transcript as a percentage of the total for all five genes. The top two panels show 26 isolates of 34 that fall into group I (8 other isolates in group I are shown in Supplementary figure), with the top panel corresponding to subgroup Ia and the second to subgroup Ib. The bottom panel shows the six isolates that fall into group II expressing *Rh2b* as a predominant transcript, and the two isolates in group III expressing *Rh4* as the most abundant transcript. (B) UPGMA cladistic analysis of the differences in expression profile among the isolates, showing branch points for group I (as well as subgroups Ia and Ib), and groups II and III. The branching between the major groups was the same in a neighbour-joining tree (data not shown). Isolates numbered 1–16 were cultured fresh after collection in 2003–2004, and isolates 17–42 were cultured in 2004 after having been cryopreserved from blood collected in 1994–1996; M: mild malaria cases, S: severe malaria cases; an asterisk (*) denotes an isolate that apparently had a single clone, the remainder being multiple clone infections except for isolate 2 from which there was no DNA available.

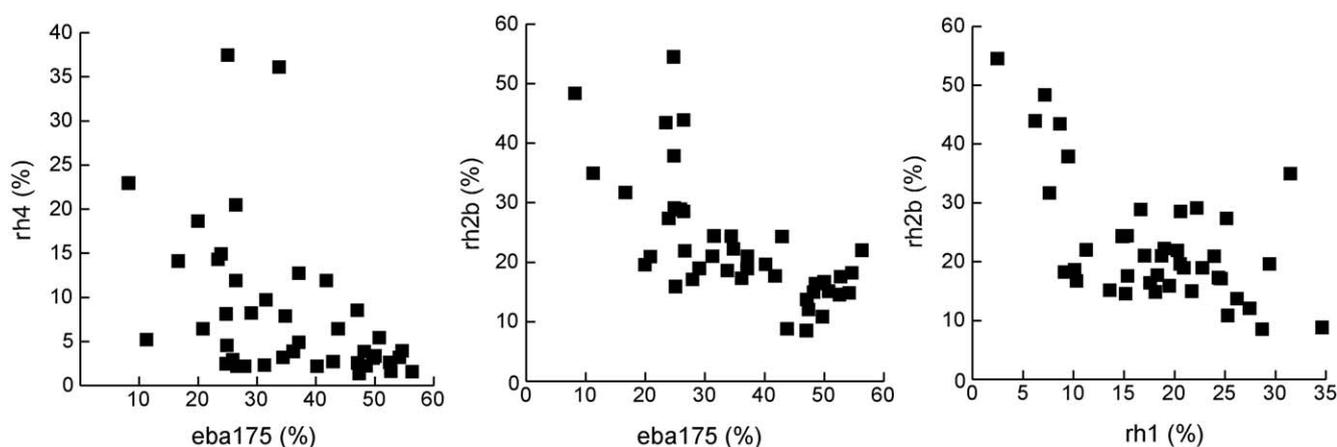


Fig. 2. Scatter plots showing the three highly significant rank correlations among levels of expression of particular *Rh* genes and *eba175*, among the 10 pairwise correlations for the five genes. (A) High levels of *eba175* are associated with low levels of *Rh4* ($\rho = -0.526$, $P < 0.001$). (B) High levels of *eba175* are associated with low levels of *Rh2b* ($\rho = -0.717$, $P < 0.001$). (C) High levels of *Rh1* are associated with low levels of *Rh2b* ($\rho = -0.479$, $P < 0.001$). Of the other seven pairwise correlations among transcript levels, one was weakly positive and one weakly negative and five were not significantly different from random (see text).

3.3. Tests for associations between gene expression levels, clinical disease status and other variables

The relative level of each transcript was compared among isolates and tested for association with different variables. There was no overall difference between isolates from the 22 patients with severe malaria or the 20 with mild clinical malaria for any of the individual transcripts (Mann–Whitney U tests, $P > 0.05$). Consistent with this, the severe and mild isolates were apparently randomly scattered among the different expression profile groups in the cladistic analysis (Fig. 1). There was also no association between parasitaemia in the patients and relative levels of expression of any of the genes.

Overall, parasite isolates that were cultured fresh *ex vivo* had slightly lower levels of *Rh1* than isolates that had been cryopreserved before culturing (Mann–Whitney U test, $P < 0.037$), although there was no difference for any of the other transcripts. This difference was also reflected in the observation that only fresh isolates were present in expression profile group II that has low levels of *Rh1* (Fig. 1).

Thirteen of the fresh isolates were tested for associations between relative amounts of gene expression and parasite growth and invasion phenotypes (the remaining three fresh isolates did not yield adequate numbers of reinvaded cells for counting). Invasion phenotype was determined by measuring the ability of merozoites to invade donor RBCs treated with neuraminidase, trypsin and chymotrypsin compared to the ability of invading untreated RBCs. Parasite growth was estimated as the first generation multiplication rate. Of the 20 correlation tests explored (four invasion or growth phenotypes against five gene transcripts), there were two significant correlations: a positive correlation between *eba175* levels and sensitivity of invasion to neuraminidase treatment (Spearman's $\rho = 0.582$, $P = 0.037$), and a positive correlation between *Rh2b* levels and parasite multiplication rate ($\rho = 0.654$, $P = 0.015$).

The presence of multiple clones of *P. falciparum* in the isolates, that could potentially give mixed or 'hybrid' phenotypes,

was assayed by amplification of the highly polymorphic repeat sequences in *msp1* and *msp2* from all isolates (except fresh isolate 2 from which no material remained for DNA extraction). The mean number of distinct genotypes detected per isolate ranged from one to six, with a mean of 2.2 per isolate, and 32% (13/41) were apparently single clone infections. Although the single clone isolates were a minority of the total, they were represented in all the gene expression clusters (marked with asterisks in Fig. 1 and Supplementary figure), indicating that the major clusters are not artifacts of superimposed phenotypes. However, some of the expression profiles of mixed genotype isolates are likely to reflect such superimposition. This may be the case for the eight samples shown in Supplementary figure (belonging to group I but not segregating into subgroup Ia or Ib), all except one of which are mixed genotype infections.

3.4. Testing for candidate gene copy number amplification

Rh1 gene copy number (shown to vary among culture-adapted isolates [9]) was assayed in 36 of the isolates for which adequate DNA remained for analysis. Copy number was compared to the single locus genes *ama1* and *seryl tRNA synthase*, and control genomic DNA from cultured *P. falciparum* isolates was also assayed (3D7 which has a single copy of *Rh1*, and FCB-1 which has been previously estimated to have four copies [9]). In the assays here, 3D7 was shown consistently to have a single copy and FCB-1 to have multiple copies (approximately seven in most assays). All of the 36 field isolates tested had an estimated *Rh1* copy number of 1 (none had a reproducible estimate approaching 2, or more than that for the 3D7 control) (data not shown). Following this, 10 isolates were randomly chosen for analysis of gene copy number of the other *Rh* genes and *eba175* (there is no previous evidence of genomic copy number amplification in culture-adapted isolates for these genes, although isolate D10 has a deletion of gene *Rh2b* [6]). None of the isolates tested here had an estimated gene copy number different from 1.

3.5. Preliminary assay of additional candidate genes

After the analysis showed that the abundance of particular gene transcripts varied in relation to each other and to the gene encoding the principal ligand *eba175*, it was considered that the expression of additional genes could also vary between isolates. The very limited schizont-stage RNA material from the isolates studied here was mostly exhausted, but sufficient remained from 14 isolates to allow a preliminary assay of variation in two other *eba* genes (*eba140* and *eba181*) alongside *eba175* (the other paralogues ψ *eba165* and *ebi-1* are putative pseudogenes and were not tested). Among these isolates, variation in relative transcript levels measured for *eba140* and *eba181* was slightly less than that for *eba175* (variance/mean ratios of 2.20, 1.44, and 3.74, respectively).

4. Discussion

These results indicate that gene expression differences that have been shown to correlate with invasion phenotypes of culture-adapted and transgenic *P. falciparum* lines are reflective of differences that occur naturally. Among the Kenyan isolates studied here, high relative levels of *eba175* expression were very strongly negatively correlated with *Rh4* levels, in agreement with the upregulation of *Rh4* in parasite lines that have the *eba175* gene knocked out, and in lines that are selected for sialic acid-independent invasion [8]. High levels of *Rh1* were strongly negatively correlated with *Rh2b* expression, which is in agreement with the pattern of variation among different culture-adapted laboratory isolates [6]. There was also a strong negative correlation between *eba175* and *Rh2b* gene expression, as might be expected because the receptors of these respective ligands have opposite sensitivities to enzyme treatment (the EBA175 receptor Gyp A is trypsin and neuraminidase sensitive, whereas the Rh2b receptor Z is trypsin and neuraminidase resistant).

The fact that there were no significant differences in the expression profiles of the five candidate genes between the 22 severe malaria and the 20 mild malaria isolates may have been expected, as these numbers give very modest statistical power. This indicates that parasites in severe malaria infections are not a highly restricted phenotypic subset, and a much larger case–control study will be needed to test for any disease associations. It is not known why *Rh1* transcript levels were lower in fresh isolates than cryopreserved ones, so that expression profile group II consisted only of fresh isolates. It is unlikely that cryopreservation of ring stage parasites from patients would alter the relative level of transcription of genes at the schizont stage, so it could be merely a chance association. Alternatively, it is possible that there were underlying differences in the expression profiles of *P. falciparum* isolates collected at the Kilifi District Hospital in 1994–1996 compared with 2003–2004.

For most of the isolates cultured here for gene expression analysis, there was insufficient volume of blood available for erythrocyte invasion assays to be performed. However, thirteen of the fresh isolates were included among a panel of isolates from

Kenya and Mali that were tested for parasite growth and invasion [18]. Although the number is too low to allow a highly informative analysis of gene expression in relation to phenotype, correlation coefficients were explored for 20 preliminary tests (relative amounts of each of the five candidate transcripts versus four culture phenotypes). This yielded two significant correlations, a positive correlation between *eba175* levels and sensitivity of invasion to neuraminidase treatment of target erythrocytes, and a positive correlation between *Rh2b* levels and parasite multiplication rate. These correlations could have occurred by chance and need to be tested for in a larger study, but it is worth noting that the first is consistent with the known role of EBA175 in binding to glycophorin A [21,22] which is the main mechanism underlying the neuraminidase sensitive invasion phenotype in culture-adapted lines [3] although there are others that are also used in some lines [4,23–25].

The three major expression profile clusters for the candidate genes studied here are quite clearly defined, with the first and largest being associated generally with higher levels of *eba175*, and the other two being associated with higher levels of *Rh2b* and *Rh4*, respectively. Although this clustering scheme helps to describe the data from these field isolates, further work is needed to develop the definition of expression profiles. The largest cluster here (group I) is diverse, and might be composed of different subgroups that need to be more clearly defined (e.g. subgroups Ia and Ib which appear to differ in the relative amount of *eba175* transcript). The inclusion of additional candidate genes involved in invasion phenotypes might qualitatively alter the profiles if expression of these varies among isolates. A preliminary analysis of *eba140* and *eba181* here indicated that their expression varied less among different isolates than *eba175*, but these are still candidates for further study, as is the newly identified *Rh5* [2]. Microarray analysis of whole transcript profiles of schizont stage parasites should help identify additional genes that differ in their expression among isolates, and some of these may also be added into the panel of invasion phenotype candidate genes.

This is the first characterization of gene expression in malaria parasite field isolates focusing on ligands involved in erythrocyte invasion, and shows the existence of distinct expression profiles. Whether the different profiles are associated with invasion phenotypes or severity of disease, and whether they are selected by acquired immune responses, remains to be elucidated by larger studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2006.05.014.

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