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Integrative analysis of intraerythrocytic differentially expressed transcripts yields novel insights into the biology of *Plasmodium falciparum*

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Abstract

Background: The intraerythrocytic development of *Plasmodium falciparum*, the most virulent human malaria parasite involves asexual and gametocyte stages. There has been a significant increase in disparate datasets derived from genomic and post-genomic analysis of the parasite that necessitates delivery of integrated analysis from which biological processes important to the survival of the parasite can be determined.

Methods: In order to resolve genes associated with stage differentially expressed transcripts, we have developed and implemented an integrative approach that combines evidence from *P. falciparum* expressed sequence tags (ESTs), genomic, microarray, proteomic and gene ontology data.

Results: A total of 143 gametocyte-overexpressed and 51 asexual-overexpressed transcripts were identified. A subset of 74 genes associated with these transcripts showed evidence of stage-correlated protein expression, of which 53 have not been experimentally characterised. Our study has revealed (1) possible regulatory mechanisms in malaria parasites' gametocyte maturation, (2) correlation between EST and microarray data for a *P. falciparum* gene family to present unique EST-derived information, (3) candidate drug and antigenic targets on which computational and experimental studies can be performed, and (4) the need for more empirical studies on gene and protein expression in malaria parasites.

Conclusion: Applying different domains of data to the same underlying gene set has yielded novel insights into the biology of the parasite and presents an approach to appraise critically the data quality of post-genomic datasets from malaria parasites.

Background

Pathogen bioinformatics have been developed and applied as a vehicle to discover novel genes and the search for virulence-associated genes combining approaches that assay gene expression, adaptive evolution and gene transfer [1-3]. In this study, layers of data about *Plasmodium fal-*

ciparum, obtained with gene transcript and genome sequencing as well as gene and protein expression profiling technologies, were integrated to reveal insights into previously undiscovered regulation during intraerythrocytic development. Genes that merit further analysis are described. This integrative approach uses an evidence-

based assessment of disparate datasets similar to gene structure prediction approaches that rely on accumulation of evidence such as similarity to known genes, nucleotide compositional features, intron/exon boundaries and promoter sequences [4].

The high malaria burden in Africa [5,6] necessitates increased efforts to understand the biology of the pathogen with a view to discovering new drugs, candidate vaccines and diagnostics, as well as improving existing ones. The publication of the genomes of the human malaria parasite *P. falciparum* and the rodent malaria parasite *Plasmodium yoelii* as well as ongoing sequencing projects of other *Plasmodium* species presents new opportunities to achieve the above-mentioned goals [7-9]. In addition, there have been efforts to obtain and analyse on a large-scale, gene expression profiles (transcriptome) of *Plasmodium* species using Expressed Sequence Tags (ESTs) [1,10-13], full length cDNAs [14], Serial Analysis of Gene Expression (SAGE) [15,16] and microarrays [17-19]. Protein expression profiles (proteome) on particular stages of the *P. falciparum* life cycle are also available [20,21].

The random single-pass sequencing of a cDNA library to generate short (200–500 bp) nucleotide sequences that tag an expressed gene sequence is an established method of gene discovery [22,23]. EST gene indices are generated by computer-based methods to organise these tags by assigning them into groups to remove redundancies and yield reconstructed transcripts that represent consensus sequences of each group [22,24,25]. These indices are being used to understand the complexity of the human genome, especially in providing information on alternative transcripts, non-translated transcripts, truly unique genes and extremely short genes that will complement the genome data [25]. The availability of the complete genome of *P. falciparum* 3D7 makes it possible to provide similar information for the parasite. In fact, additional EST and full-length cDNA sequences are required to improve the current annotation and verify predicted genes [7]. EST sequencing projects on *Plasmodium* have identified novel genes [1,10,13] but only limited analyses have been performed on ESTs for coordinate and differential gene expression [13].

Plasmodium ESTs from a variety of cDNA libraries are available in the GenBank EST database (dbEST). As of February 2003, 11 libraries comprising of nine asexual, one sporozoite and one gametocyte were available in dbEST. ESTs from some of these libraries have been indexed [1,10,13,26]. Microarrays, mRNA differential display and EST-based analysis have been used to study transcriptional differences between asexual and gametocyte stages of *P. falciparum*, revealing stage-specific genes [13,17,27]. These studies were done prior to the publica-

tion of the genome sequence of strain 3D7. Furthermore, in the case of Li and colleagues [13], the functional annotation was selective. An EST-based analysis with an improved functional annotation that combines the automated annotation from *P. falciparum* gene indices and the curated annotation in the *Plasmodium* Genome Database (PlasmoDB) [28] is needed. In addition, integration of proteomic data with such analysis has been recognized as an important component in drug target identification and validation in the human genome [29].

The number of ESTs used to generate a consensus sequence in a gene index can provide a rough estimate of the mRNA abundance in the tissue or cell of origin [23]. Furthermore, statistical tests have been developed to identify genes that are differentially expressed (significantly overexpressed) in a particular tissue compared to one or more other tissues [30,31]. The differences in EST counts have been applied to understand gene expression in different metabolic pathways, tissues or stages [32-34]. These differences appear to correlate with biology of the tissue or stage under investigation. Microarray and SAGE methods are more narrow but sensitive for differential gene expression studies and can be used to validate broader EST-based analysis [13].

The life cycle of *P. falciparum* involves stages in the female anopheline mosquito vector and stages in the human host [35]. The parasite goes through pre-erythrocytic and intraerythrocytic stages in the human host. The pre-erythrocytic stage involves invasion and growth within liver cells, whereas the intraerythrocytic cycle is a multi-stage process, which includes differentiation into asexual stages (rings, merozoites, trophozoites and schizonts) as well as sexual stages (male and female gametocytes). The clinical symptoms of malaria are produced primarily as a consequence of the asexual life cycle, while the sexual cycle, which can be divided into early (I-II) and late (III-V) gametocyte stages [36], is necessary for the development of the parasite in the mosquito. The intensive research on gene expression in the asexual stage compared to gametocyte stage can be inferred from the number of cDNA libraries deposited in the dbEST as mentioned above. The late (mature) stage gametocyte cDNA library (ID:10054) should contain transcripts important for gametocyte maturation and also formation of gametes and fertilization [37]. The availability of a cDNA library of 3D7 (ID:9765) asexual mixed stage (rings, trophozoites and schizonts) and genome data from the same strain presents an opportunity to determine differentially expressed transcripts between the two libraries.

Transcription and translation in malaria parasites is complex and characterized by features such as multiple transcripts, antisense transcripts, stage-specific transcripts,

chromosomal clusters encoding co-expressed proteins, unspliced mRNA, gene family member-specific expression and translational control [20,38,39]. These features contribute to parasite fitness and ability to undergo a complex life cycle. Understanding the role of these features in the regulation of important intraerythrocytic biological processes can deliver new tools for malaria control. For example, a proportion of genes involved in glycolysis, proteolysis and apicoplast targeting of nuclear encoded genes are thought to be regulated during the transition from asexual to sexual stages [7,40]. The integration of data from EST sequencing with those from genomic, microarray and proteomic technologies could provide insights into molecular mechanisms that contribute to the regulation of these processes.

The significant increase in disparate datasets from genome sequencing and post-genomic analysis of *P. falciparum* necessitates delivery of integrated analysis from which biological processes important to the survival of the parasite can be determined. The integrated approach developed has identified stage-overexpressed genes with computational and experimental evidence to support their functional analysis. Furthermore, the approach is demonstrated as a means to appraise critically the data quality of the increasing number of post-genomic datasets from malaria parasites.

Methods

Integrative analysis approach

The integrative analysis approach that was used to combine genomic, expressed sequence tag, microarray, proteomic and gene ontology data from *P. falciparum* 3D7 is presented in Figure 1. The starting integrative criterion was significant overexpression of a transcript in a stage relative to the other stage. Criteria used and their acceptable ranges are presented in Table 1.

Expressed sequence tags and transcript reconstruction

Expressed Sequence Tags derived from *P. falciparum* 3D7 mixed asexual stage (dbEST ID: 9765) and gametocyte (III-V) stages (dbEST ID: 10054) cDNA libraries were retrieved using Sequence Retrieval System (SRS) version 7.02 from EMBL database (Release 74, March 2003). These sets of ESTs were sequenced by Washington University *Plasmodium* EST Project [13]. A total of 15,126 ESTs consisting of 11,872 asexual and 3,254 gametocyte ESTs were downloaded. Transcript reconstruction of these ESTs was performed using stackPACK clustering system version 2.2 [22,24] as described previously for reconstructing *Plasmodium* transcripts [1]. Briefly, the process starts with removal of artifactual sequences such as repeats and vector sequences. The "clean" sequences are grouped using a loose clustering approach into clusters and the clusters assembled into contigs. The alignments of sequences that

make up these assembled clusters are analysed to produce consensus sequences of maximal length representing the reconstructed transcripts. stackPACK was chosen for its ability to provide extended consensus sequences [41] (Hide et al. in preparation). Clusters containing only a single sequence are called singletons. A gene index, manufactured by such a method, is therefore a non-redundant representation of a set of reconstructed gene fragments that approximates to the best available representation of genes for that organism. The clustering was unsupervised in that known sequences such as mRNA, full-length cDNA, previously reconstructed ESTs or exon constructs were not used to guide the process. This type of clustering was required to provide valid input data for the software used to calculate the differential expression statistics applied in this study.

Differential gene expression analysis

Audic-Claverie (AC) and the Chi-square (χ^2) 2×2 statistical tests for differential gene expression were used to identify stage-overexpressed transcripts. These pairwise tag statistics are based on EST counts of contigs (assembled clusters) with at least five ESTs since for a 95% confidence interval, the first value that is significantly different from 0 is 5 [30,32].

The calculation of these statistics was implemented with the web version of IDEG6 software; <http://telethon.bio.unipd.it/bioinfo/IDEG6/> with a significance threshold of 0.05 [31]. A suite of PERL scripts was written to extract EST counts from output of stackPACK 2.2 and present the input dataset in the format required by IDEG6. Data extracted from the output file of IDEG6 were (1) contig description; (2) observed and normalised EST counts from the two libraries; and (3) probability that a transcript is differentially expressed as represented by *P*-values for the two tests. Transcripts for which the *P*-values for both statistics were less than 0.05 were taken as differentially expressed. Since these statistics determined transcripts differentially expressed, the terms asexual-overexpressed and gametocyte-overexpressed were used for transcripts (or genes) with significant overexpression in mixed asexual stage and late stage gametocytes respectively.

Protein expression profiles and functional annotation of transcripts

Annotated protein predictions (release 4.0) of the whole genome sequence of *P. falciparum* 3D7 was obtained from the PlasmoDB website; <http://www.plasmodb.org>. A total of 5,334 predicted protein sequences were obtained. The overview page for each gene was retrieved using wget and saved as a Hypertext Markup Language (HTML) file on a local computer to allow ease of manipulation without accessing the database over the Internet. A PERL script was

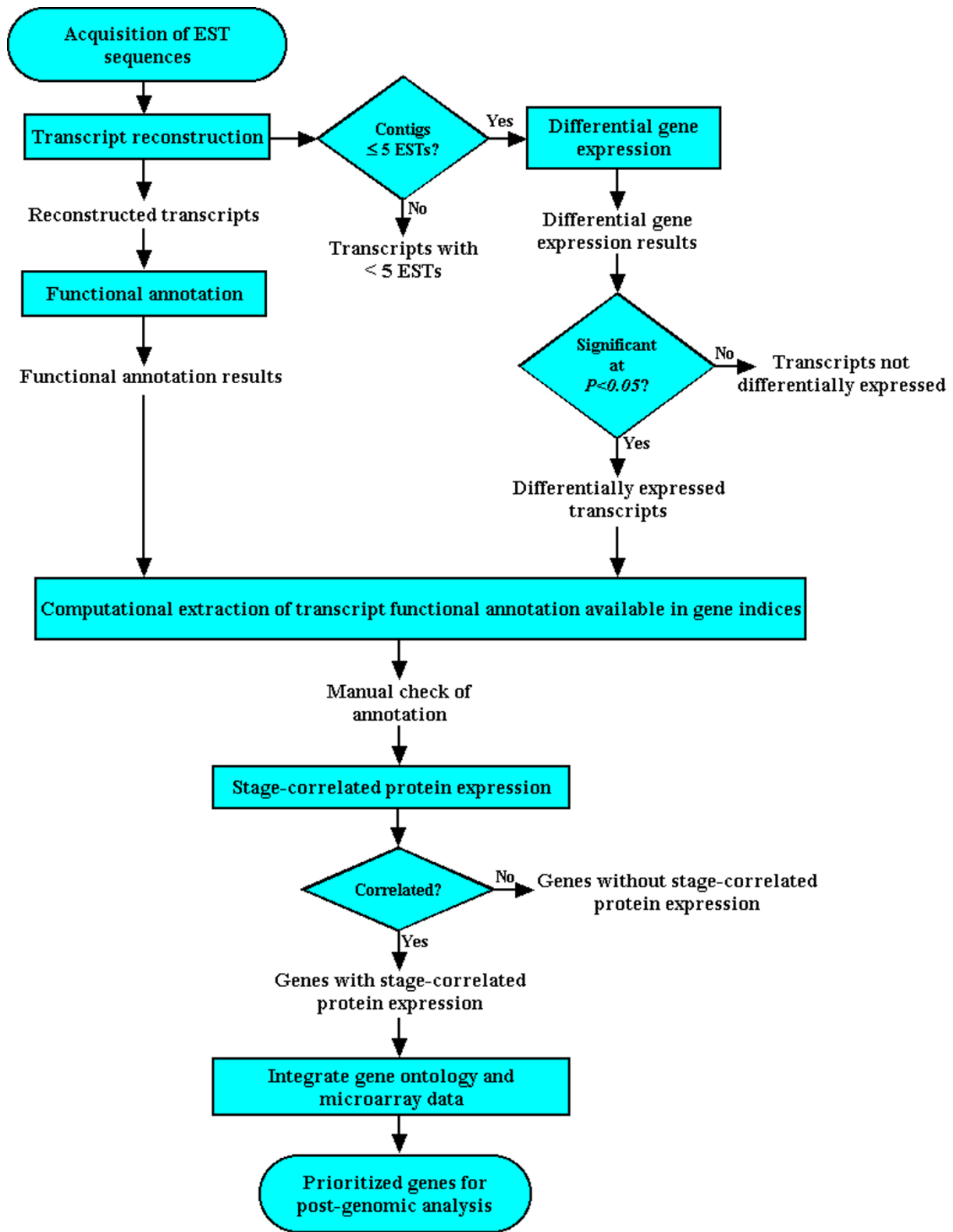


Figure 1
Simplified flowchart of integrative analysis of *Plasmodium falciparum* data. Flowchart symbols: rounded rectangle, start or end; rectangle, process; diamond, decision.

Table 1: Threshold values for steps in integrative analysis of *Plasmodium falciparum* data

Criterion and acceptable range
Reconstructed transcript derived from minimum of 5 ESTs
Agreement of pairwise differential expression statistics at $P < 0.05$
Maximum BLASTX E-value of 10^{-10} against predicted proteins
Correlation of functional annotation with <i>Plasmodium falciparum</i> gene indices
Evidence that protein is expressed in same stage as gene
Gene Ontology classification: proteolysis, glycolysis or localised to plastid
Microarray: Published data on a gene family

used to query each page for the words sporozoite, merozoite, trophozoite or gametocyte preceded by an apostrophe (') followed by a specific text as for the gametocyte; 'gametocyte stage peptide fragment(s) detected by mass spectrometry'. A match of this text was taken as evidence of expression and protein expression at the stage was assigned 1 or else 0 for no evidence. Thus, a 4-digit binary accession that indicates evidence for expression in sporozoite, merozoite, trophozoite and gametocyte is used to represent the 15 protein expression profiles presented by Florens et al. [20] and an additional accession for lack of evidence in all stages (0000).

Reconstructed transcripts were annotated on the basis of similarity searches using NCBI BLASTX version 2.2.1 against predicted proteins of *P. falciparum* 3D7. Statistical significance cut-off was set at an E-value of 10^{-10} following that of Carlton et al. [1]. Since an unsupervised clustering was performed, to support the functional annotation, the annotations obtained were correlated with the TIGR *P. falciparum* Gene Index; <http://www.tigr.org/tdb/tgi/pfgi/> (Version 6.0, Release Date – January 11, 2003) and the Apicomplexan EST Database (ApiESTDB); <http://www.cbil.upenn.edu/paradbs-servlet/>. Both these indices were generated with supervised clustering. The correlation was done by computational extraction of associated annotation of the TIGR Tentative Consensus (TC) followed by manual checking to determine if the annotation obtained in our analysis was identical to that of the TIGR TCs. This was done for only differentially expressed contigs. If the annotations were not identical, the reconstructed sequence was excluded from further analysis. ApiESTDB was consulted when additional support was required to make a decision.

Mining gene ontology annotation associated with transcripts

Genes classified as being involved in glycolysis (GO:0006096), proteolysis (GO:0006508) or targeted to the plastid (GO:0009536) were retrieved by searching PlasmoDB gene overview page for the respective GO identification (ID) number in a similar way as described for

the protein expression profile except the search text was the respective GO ID preceded by the greater than sign (>) for example >GO:0006096. This text limits the search to the Gene Ontology section of the gene overview page. The number of genes retrieved was: 20 for glycolysis, 98 for proteolysis and 553 for plastid component. This corresponds to values obtained from the web-based PlasmoDB query page.

Correlation of EST-based abundance with microarray expression levels

The numbers of ESTs used to generate a reconstructed sequence were retrieved from the FASTA sequence description line of all reconstructed sequences generated by stackPACK 2.2. The levels of expression or average signal intensities obtained from microarray experiments on the serine repeat antigen (SERA) gene family of *P. falciparum* [19,42-44] were used to compare the levels of expression obtained using ESTs. This gene family is characterised by a cysteine proteinase framework [39] and was selected because its members are annotated as being involved in proteolysis. Published microarray studies on this family have been obtained that facilitated comparative analysis with EST data.

Results

Transcript reconstruction and functional annotation of transcripts

Transcript reconstruction using stackPACK 2.2 resulted in 1,760 contigs and 3,391 singletons. A total of 569 transcripts had an EST count of at least five ESTs. Functional annotation by similarity searching was performed for all reconstructed transcripts. A total of 210 transcripts that were differentially expressed were manually checked for correlation with TIGR and/or ApiESTDB *P. falciparum* gene indices. This process yielded 194 transcripts with correlated functional annotation.

Differential expression transcripts and protein expression profiling

The majority of the stage-overexpressed transcripts were from the late gametocyte stage. However, the mixed

Table 2: Summary of functional annotation and protein expression of *Plasmodium falciparum* transcripts

Transcripts	Number
Differentially expressed	210
Correlated functional annotation	194
Stage-overexpressed	
Mixed asexual stage	51
Late stage gametocyte	143
With significant match to predicted proteins	
Mixed asexual stage	48
Late stage gametocyte	128
Correlated protein expression	
Mixed asexual stage	40
Late stage gametocyte	38

asexual stage had the highest percentage (83%) of genes with evidence of protein expression in the same stage (stage-correlated protein expression) compared to 31% for the late gametocyte stage. The observations are summarised in Tables 2 to 5. The 194 transcripts differentially expressed between the two libraries consisted of 51 from the mixed asexual stage and 143 from the late gametocyte stage. The complete list with transcript identification used in this study, correlated transcripts in the TIGR *P. falciparum* gene index, gene locus name, gene product description, representative EST or ESTs (for genes with representation from both libraries), observed and normalized EST counts for the two stages, as well as protein expression profile, are presented in the additional files 1 and 2 for mixed asexual stage and late gametocyte stage respectively. A list of stage-overexpressed transcripts that match those of Li et al. [13] is presented in additional file 3.

A total of 128 gametocyte-overexpressed and 48 asexual-overexpressed transcripts had a significant match with the predicted *P. falciparum* 3D7 proteins. Seventy-four genes (40 asexual-overexpressed, 34 gametocyte-overexpressed) showed evidence of stage-correlated protein expression (Tables 3 and 4). The well-studied S-antigen (PF10_0343) is one of the 8 asexual-overexpressed genes without stage-correlated protein expression. Four gametocyte-overexpressed genes (PFB0730w, PFI1210w, PF10_0115 and PFL0105w) had more than one reconstructed transcript. Multiple transcripts were generated when the reconstructed transcripts associated with a gene are not contiguous, and thus were not assembled into the same contig. Fifty-three of the 74 genes were classified as novel in that either the description of the gene product is labelled hypothetical protein or have the word putative.

In order to identify gametocyte-overexpressed genes that also have stage-correlated protein expression in the pro-

teomics data of Lasonder et al. [21], the spreadsheet file containing 1,289 unique malaria proteins from that study was processed to yield a 3-digit binary accession representing evidence for protein expression of genes in trophozoites/schizonts, gametocytes and gametes. Fifteen of the 34 gametocyte-overexpressed genes were detected by both proteomic analyses (Table 6). Our analysis points to the need to clarify potential confusion in the annotation of the sexual stage specific protein precursor or Pfs16 (PFD0310w), a known marker for the earliest events of sexual differentiation [45]. The locus name (PF11_0318) of another gene, PF16, may be assigned to this gene [21]. PF16 has sequence similarity to a sperm flagella protein localized to the central pair of the axoneme. The gametocyte-overexpressed gene identified in this study was confirmed to be Pfs16 and not PF16 by the identical functional annotation of the associated consensus sequence from this study and that in the TIGR *P. falciparum* gene index.

The identified asexual-overexpressed genes that have been experimentally characterised have known roles in protein degradation, purine salvage, rhoptry biogenesis and protein trafficking, schizont rupture, merozoite invasion, phospholipid biosynthesis, nuclear metabolism, oxidative stress defense, cell proliferation and membrane biogenesis.

Mining gene ontology annotation associated with transcripts

Glyceraldehyde-3-phosphate dehydrogenase (PF14_0598) and ATP-dependent phosphofructokinase (PF11_0294) are two of 20 genes known to be involved in glycolysis. They demonstrate differential expression and show evidence of stage-correlated protein expression.

Microarray average intensities [19] available in PlasmoDB for PF11_0294 support its gametocyte-overexpression

Table 3: Asexual-overexpressed *Plasmodium falciparum* transcripts

Transcript ^a	TIGR Tentative Consensus ^b	Gene locus name ^c	Description of gene product	Representative EST(s) ^d
cn672	TC6879	PF10265c	rhoptry protein, putative	BI670632
cn1243	TC6890 TC6891	PFL1385c	101 kd malaria antigen	BI670667
cn656	TC6894	PF11_0098	endoplasmic reticulum-resident calcium binding protein	BI670528 BM274707
cn346	TC6883 TC6884 TC6885	PF14_0598 ^e	glyceraldehyde-3-phosphate dehydrogenase	BI670581 BM273393
cn659	TC6886 TC6887	PFB0340c ^g	cysteine protease, putative	BI670678
cn646	TC6895	PF14_0102	rhoptry-associated protein I	BI670673
cn1292	TC6896	PF10875w	Heat shock protein	BI670644
cn634	TC6897 TC6898 TC8065	MAL13PI.214	phosphoethanolamine N-methyltransferase, putative	BI670572
cn1258	TC6900	PF11445w	hypothetical protein	BI670690
cn1175	TC6899	PFC0120w	Cytoadherence linked asexual protein, CLAG	BI670808
cn637	TC6921	PFE0165w	actin depolymerizing factor, putative	BI813965 BM274236
cn1246	TC6922	MAL8PI.142 ^g	proteasome beta-subunit	BI670563
cn628	TC6926	PF10_0203	ADP-ribosylation factor	BI814382
cn1338	TC6943	PF14_0141	ribosomal protein L10, putative	BI670722
cn1375	TC6945	MAL7PI.77	hypothetical protein	BI814179
cn1569	TC6954 TC6955	PFE0915c	proteasome subunit beta type I	BI670682
cn1255	TC6969 TC7520	PFB0445c	helicase, putative	BI670715
cn604	TC6958	PFL0210c	eukaryotic initiation factor 5a, putative	BI670597
cn1249	TC6970	PF07_0054	histone h2b, putative	BI670668
cn1465	TC6959	PF14_0368	2-Cys peroxiredoxin	BI670633
cn581	TC6975	PF14_0543 ^f	hypothetical protein, conserved	BI814501
cn1219	TC6956	PF10_0345	merozoite surface protein-3	BI670568
cn1339	TC6992	PFL1420w	macrophage migration inhibitory factor homolog, putative	BI815759
cn1396	TC6971	PF10_0121	hypoxanthine phosphoribosyltransferase	BI814714
cn567	TC6917	PF10_0268	merozoite capping protein-I	BI670775
cn1555	TC7001	PF10155c	ras family GTP-ase, putative	BI814010
cn561	TC7038	PF10_0016	acyl CoA binding protein, putative	BI815304
cn1165	TC7015	PFD0240c	hypothetical protein	BI816061
cn1379	TC7007	PF07_0087 ^f	hypothetical protein	BI813959
cn1475	TC6914	PF11090w	s-adenosylmethionine synthetase, putative	BI813864
cn1811	TC6989 TC6990	PF14_0323	calmodulin	BI814267
cn564	TC6993	PFE1050w	adenosylhomocysteinase(S-adenosyl-L-homocysteine hydrolase)	BI814536
cn613	TC7023 TC8311	PFB0490c	hypothetical protein	BI815328
cn1485	TC7032	PF13_0228	40S ribosomal subunit protein S6, putative	BI670560
cn1681	TC7025	PF13_0328	proliferating cell nuclear antigen	BI813993
cn558	TC7018	PF14_0678	exported protein 2	BI670646
cn1605	TC6904	MAL13PI.130	hypothetical protein	BI814223
cn1997	TC7030	PFE0660c	uridine phosphorylase, putative	BI814451
cn557	TC7036	PF13_0092	cholinephosphate cytidyltransferase	BI814410
cn1368	TC7086	PF14_0569	hypothetical protein	BI814420

^a Transcript generated by stackPACK 2.2. ^b TIGR Tentative Consensus correlated with transcript available at <http://www.tigr.org/tdb/tgi/pfgi/>. ^c Gene can be viewed at <http://www.plasmodb.org>. ^d EST can be retrieved at <http://www.ncbi.nlm.nih.gov>. ^e Gene involved in glycolysis. ^f Apicomplast-targeted gene. ^g Gene involved in proteolysis.

when compared to a closely related gene, PF10755c that also codes for a phosphofructokinase and shows protein expression in intraerythrocytic stages [20,21]. The microarray expression values for PF10755c in trophozoite and schizont stages are 17,223.33 and 7,894 respectively in contrast to ~1,600 in both stages for PF11_0294. Inspection of the predicted protein features of PF11_0294 revealed the presence of two protein domains: gonadotro-

pin-releasing domain, GnRH (Pfam ID: PF00446) and laminin N-terminal (Domain VI) (Pfam ID: PF00055). These domains are found in proteins that are extracellular and have a role in regulation of germ cell development.

PFB0340c, a cysteine protease and member of the SERA gene family was significantly overexpressed in mixed asexual stage. Other genes in the SERA family for which EST

Table 4: Gametocyte-overexpressed *Plasmodium falciparum* transcripts

Transcript ^a	TIGR Tentative Consensus ^b	Gene locus name ^c	Description of gene product	Representative EST(s) ^d
cn298	TC6923 TC7279 TC9304	PFD0310w	sexual stage-specific protein precursor	BI814617 BM273325
cn156	TC6995	PFL0795c	hypothetical protein	BI813971 BM273682
cn144	TC7077	PF11_0525 ^f	hypothetical protein	BM273367
cn369	TC6974	PF10_0264	40S ribosomal protein, putative	BI814069 BM273547
cn57	TC7312 TC7511	PFL2420w	hypothetical protein	BM273440
cn271	TC6963	PFB0730w	DNA helicase, putative	BM273418
cn291	TC6911	PF07_0029	heat shock protein 86	BI670622 BM273491
cn43	TC6936	PFL2215w	actin	BM273378
cn105	TC7084	PF07_0061	hypothetical protein	BI936117 BM273354
cn168	TC6963	PFB0730w	DNA helicase, putative	BM273308
cn178	TC6987	PF11210w	hypothetical protein	BM274237
cn337	TC7315	PF08_0081	hypothetical protein	BM274748
cn404	TC7057	PF10_0115	QF122 antigen	BM273319 BQ596378
cn46	TC7235	PFL0105w	hypothetical protein	BM273988 BQ577236
cn246	TC7159	PF14_0359	hypothetical protein, conserved	BI814120 BM273571
cn60	TC7496	PF10_0328	hypothetical protein	BM273370
cn155	TC7437	PF11_0294 ^e	ATP-dependent phosphofructokinase, putative	BM273524
cn269	TC7203	MAL6PI.306	hypothetical protein	BI815038 BM273934
cn347	TC6987	PF11210w	hypothetical protein	BM273395
cn19	TC7561	MAL13PI.148	<i>P. falciparum</i> myosin	BM274131
cn683	TC7619	PFD0235c	hypothetical protein	BM274865
cn833	TC7170	PFL1070c	endoplasmic homolog precursor, putative	BI670681 BM273857
cn71	TC6893	PFL0105w	hypothetical protein	BM274046
cn93	TC7763	PF11_0460	hypothetical protein	BM273313
cn165	TC7103	PF13_0165	hypothetical protein	BI670714 BM273638
cn288	TC7304	PF10_0165	DNA polymerase delta catalytic subunit	BM274252
cn685	TC7766	PF11_0331	t-complex protein 1, alpha subunit, putative	BM273631
cn717	TC7621	PF10_0115	QF122 antigen	BM273917
cn737	TC8144	PFL1395c	hypothetical protein	BM273513
cn832	TC7423	PF10460w	hypothetical protein	BM273947
cn49	TC7047	PF10_0242	hypothetical protein	BM274006 BQ597262
cn248	TC7431	PFD0685c	chromosome associated protein, putative	BI936055 BM274686
cn326	TC7394	PFC0570c	hypothetical protein	BM273462 BU496460
cn750	TC7788	PF10_0256	hypothetical protein	BM273642 BQ452171
cn945	TC7533	PFA0460c	tubulin-specific chaperone a, putative	BM273558 BQ451292
cn982	TC7573	MAL6PI.48	hypothetical protein, expressed	BI814116 BM273303
cn681	TC7652	PFE0845c	60S ribosomal subunit protein L8, putative	BM273443 BU495298
cn805	TC7301	MAL13PI.120	splicing factor, putative	BI815872 BM274487

^a Transcript generated by stackPACK 2.2. ^b TIGR Tentative Consensus correlated with transcript available at <http://www.tigr.org/tdb/tgi/pfgi/>. ^c Gene can be viewed at <http://www.plasmodb.org>. ^d EST can be retrieved at <http://www.ncbi.nlm.nih.gov>. ^e Gene involved in glycolysis. ^f Apicomplast-targeted gene.

Table 5: Distribution of protein expression profiles for *Plasmodium falciparum* stage-overexpressed genes

Gene category	Binary accession ^a	Count
Asexual-overexpressed		
With protein expression	1111, 0111, 1011, 1101, 1110, 0011, 0101, 0110, 1010, 1100, 0010, 0100	40
Without protein expression	0000, 1001, 0001, 1000	8
Gametocyte-overexpressed		
With protein expression	1111, 0111, 1011, 1101, 0011, 0101, 1001, 0001	34
Without protein expression	0000, 1110, 0110, 1010, 1100, 0010, 0100, 1000	87

^a 4-digit binary accession for protein expression evidence in sporozoite, merozoite, trophozoite and gametocyte.

Table 6: Gametocyte-overexpressed *Plasmodium falciparum* genes with correlated protein expression in two proteomic studies

Gene locus name	Description of gene product	Protein expression binary accession ^a	
		Florens et al. [20] ^b	Lasonder et al. [21] ^c
PFA0460c	tubulin-specific chaperone a, putative	0001	011
PFD0310w	sexual stage-specific protein precursor	0011	111
PFD0685c	chromosome associated protein, putative	0101	010
PFE0845c	60S ribosomal subunit protein L8, putative	0111	111
PF07_0029	heat shock protein 86	1111	111
PF10_0165	DNA polymerase delta catalytic subunit	0111	010
PF10_0242	hypothetical protein	0111	111
PF10_0264	40S ribosomal protein, putative	0111	111
PF11_0294	ATP-dependent phosphofructokinase, putative	0001	011
PF11_0331	t-complex protein 1, alpha subunit, putative	1111	111
PF11_0525	hypothetical protein	1001	010
PFL0795c	hypothetical protein	0001	011
PFL1070c	endoplasmic homolog precursor, putative	1111	111
PFL2215w	actin	1111	111
PF14_0359	hypothetical protein, conserved	0111	111

^a Evidence of expression: 0, no evidence; 1, with evidence. ^b 4-digit binary accession for protein expression evidence in sporozoite, merozoite, trophozoite and gametocyte. ^c 3-digit binary accession for protein evidence in trophozoite/schizont, gametocyte and gametes.

data were available were checked for correlation of functional annotation and their EST count retrieved. As shown in Table 7, the EST counts were variable across the gene family consistent with microarray-based studies [42-44]. There was EST evidence for expression of PFB0345c (SERA4), PFB0340c (SERA5) and PFB0335c (SERA6), the three central genes that were demonstrated to be essential for asexual stage growth [42]. The GenBank accession numbers of a representative EST from these genes are BI936220, BI815392 and BQ633262 respectively. PFB0340c showed the highest EST count and microarray intensity values during asexual development of the parasite. Furthermore, multiple contigs mapped to this gene, which may represent alternative transcripts.

Out of the 17 transcripts (four asexual and 13 gametocyte) associated with genes targeted to the apicoplast, only two genes: MAL13P1.281 and PFE0145w have similarities to known genes (glutamate-tRNA ligase and 50S ribosomal subunit protein L28). There was evidence of protein expression in at least one asexual stage for two (PF07_0087, PF14_0543) of the four asexual-overexpressed genes (Table 3). Six gametocyte-overexpressed genes showed evidence for expression in the sporozoite stage while only PF11_0525 showed evidence in the sporozoite and gametocyte stages. PF11_0525 has predicted protein motifs that indicate its likely function. The domains are IQ (calmodulin-binding motif, Pfam ID: PF00612) and LysM (lysine motif, Pfam ID: PF01476), which is a general peptidoglycan-binding module. A list of apicoplast-targeted genes with stage-overexpressed transcripts is presented in additional file 4.

Discussion

An integrative approach was used to determine genes associated with transcripts differentially expressed between mixed asexual stage and late stage gametocyte parasites. The publication of the genome sequence of two malaria parasites presents opportunities for post-genomic era malaria research including gene discovery and comprehensive understanding of gene expression [46]. The study has revealed (1) possible regulatory mechanisms in malaria parasites' gametocyte maturation, (2) correlation between EST and microarray data for a *P. falciparum* gene family to present unique EST-derived information, (3) candidate genes on which computational and experimental studies can be performed, and (4) the need for more empirical studies on gene and protein expression in malaria parasites.

A total of 569 contigs was used to determine stage-overexpression. These presents 366 more contigs than described by Li et al. [13] reflecting inclusion of new mixed asexual stage ESTs deposited after March 2002. Only 21 of the 24 significantly stage-specific transcripts identified by Li et al. [13] were among our stage-overexpressed transcripts after correlation of functional annotation. Both studies demonstrate the asexual-overexpression of the gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an important gene in the glycolytic pathway [47].

Gene and protein expression were observed, as well as protein domain evidence for specialization or adaptation of ATP-dependent phosphofructokinase (PF11_0294) for metabolic coupling of glucose utilization and maturation of gametocytes in malaria parasites. This enzyme is of

Table 7: Correlation of EST abundance and microarray intensity associated with SERA gene family

Gene (Locus name)	EST count ^a	Comments ^b Miller et al. [42]	Microarray intensity values ^c					
			Le Roch et al. [43] R	T	S	Bozdech et al. [19] T	Wu et al. [44] S Asyn	
SERA8 (PFB0325c)	-	-/+	35.3	10.4	39.3	-	-	179
SERA7 (PFB0330c) ^d	7	-/+	160.5	982.1	1298	2238	5475.83	2415
SERA6 (PFB0335c) ^e	2	+	200.7	588.6	1012.6	1695.17	4802.83	3428
SERA5 (PFB0340c) ^{e,f}	98	+	1255.4	4623.7	10265.5	13253.67	59511.17	28613
SERA4 (PFB0345c) ^e	4	+	200	496.7	1456.7	3115.17	10053.17	2273
SERA3 (PFB0350c)	-	+	87.3	341	579.7	-	6319.83	4572
SERA2 (PFB0355c)	-	-/+	185.4	219.4	399.1	-	-	1401
SERA1 (PFB0360c)	2	-/+	125.9	178.1	615.7	-	-	376

^a -, no ESTs observed. ^b Comments on gene expression: -/+, low or absent expression; +, expression confirm by RT-PCR and microarray. ^c R, Rings; T, Trophozoite; S, Schizont; Asyn, asynchronous culture; -, No expression value reported. ^d EST count of TIGR TC7227. ^e Central genes in the SERA locus that could not be disrupted in study [42]. ^f Gene with multiple transcripts, TC6886 (BI670678) TC6962 (BI814535).

major regulatory importance in *Plasmodium* and has been characterised only in *Plasmodium berghei* [48]. In addition, it has been proposed as a potential drug target in protozoan parasites [49]. Two genes (PF11_0294, PFI0755c) annotated as phosphofructokinase are present in the genome [7]. This is consistent with the fact that many key enzymes in the glycolytic pathway occur as isoenzymes [48]. Interestingly, PF11_0294 possesses a gonadotropin-releasing domain GnRH and laminin N-terminal (Domain VI) that are thought to regulate germ cell development. PFI0755c does not contain these domains.

PF11_0525 is the only apicoplast-targeted gene associated with a gametocyte-overexpressed transcript that showed stage-correlated protein expression. The fact that germ cell biology is conserved in evolution enables us to speculate on the possible roles of this protein. The calmodulin (CaM) binding site has been extensively studied in a sperm autoantigen (Sp17), which is a zona binding protein and a member of the family of CaM binding proteins that contain the IQ motif in the CaM binding domain. This domain has a regulatory role and undergoes proteolytic processing at the initiation of an acrosome reaction [50]. Some bacterial proteins such as hydrolytic enzymes contain the general peptidoglycan-binding module (LysM) and have a role in cell-wall penetration [51]. PF11_0525 does not have evidence of a bipartite peptide for apicoplast targeting and thus may be targeted via a different mechanism to the organelle or it may no longer function in the plastid.

The EST counts of the SERA gene family are comparable with the gene expression levels observed in microarray experiments. Both technologies agree that expression levels of members are variable as is expression of central genes during the asexual stage of the parasite. PFB0340c (SERA5) is the first described member of the family [39]

and is also a malaria vaccine candidate [52]. The EST counts for PFB0340c observed is consistent with high gene expression levels in trophozoites and schizonts in published microarray experiments. Specifically, Miller et al. [42] and Aoki et al. [52] observed PFB0340c to be substantially more strongly transcribed than other SERA genes.

The increasing amount of published and unpublished data from microarray, SAGE, EST and differential display on malaria parasites shows that pairwise correlation is required. Comparison of such datasets obtained from different gene expression technologies can complement less sensitive technologies, hence adding value to data generation from these methods. For example, this study provides identity of ESTs and also potential alternative transcripts that can be used to further characterize the SERA central genes. Furthermore, PFB0325c (SERA8) did not have EST evidence consistent with low or absent expression observed in the microarray studies. However, there was evidence of its expression in the sporozoite stage, indicating the gene may be functional in other stages of the life cycle as speculated by Miller et al. [42]. Large-scale comparative expression analysis of gene families in multiple malaria parasites is needed to advance the knowledge of their evolution and their role during intraerythrocytic development.

The two uncharacterized genes from which we speculate functional insights, PF11_0294 and PF11_0525, have putative orthologues in *P. yoelii yoelii* (PY05918 and PY06990 respectively) [8] and were also detected in two independent proteomic analysis as expressed in the mature gametocyte stage [20,21]. These observations strengthen the need for further studies on these genes and the possibility of studies with model malaria parasites. In general, various categories of candidate genes were pro-

vided that can be intensively studied as drug targets, antigenic targets, epidemiological or clinical markers. Eighty-seven of the 121 gametocyte-overexpressed genes did not show evidence of stage-correlated protein expression while 15 of those with such evidence were corroborated by the two proteomics studies. These corroborated genes represent a set of gametocyte-overexpressed genes with correlated transcription and translation data and thus candidates for studies on gametocyte maturation in malaria parasites. A shortlist of stage-overexpressed genes targeted to the plastid is presented to facilitate studies to understand the regulation of plastid metabolism in malaria parasites.

This study has identified the lack of correlation between gene and protein expression of the asexual-overexpressed S-antigen, consistent with observations from published proteome analysis [20]. This observation and those from the gametocyte-overexpressed transcripts as well as comparing outputs from EST clustering efforts demonstrate that our integrative approach has the utility to compare outputs of different post-genomic analysis. The analysis indicates the need for additional empirical studies on gene and protein expression in malaria parasites. Such studies could improve current understanding on discrepancies between gene and protein expression profiling data as well as the detection of proteins with unique characteristics such as proteolytic processing, post-translational modification and sub-cellular location.

Conclusions

The value of integrating a variety of datasets to unravel undiscovered regulation in biological processes during the gametocyte maturation stages of *P. falciparum* was demonstrated. Furthermore, comparative analysis of EST and microarray data was performed on the SERA gene family to advance the knowledge of their gene regulation and additional functional genomics reagents were presented to facilitate their study. Finally, the integrative approach was shown as a means to appraise critically the data quality of the increasing number of post-genomic datasets from malaria parasites.

Additional material

Additional File 1

Plasmodium falciparum asexual-overexpressed transcripts

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[<http://www.biomedcentral.com/content/supplementary/1475-2875-2-38-S1.xls>]

Additional File 2

Plasmodium falciparum gametocyte-overexpressed transcripts

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Additional File 3

Correlated stage-overexpressed transcripts in this study and that of Li et al. [13]

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Additional File 4

Plasmodium falciparum candidate genes for studies into plastid metabolism

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