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Short Communication

Duffy antigen receptor for chemokines (DARC) and susceptibility to *Plasmodium vivax* malaria

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ABSTRACT

DARC is thought to act as a key invasion receptor in *P. vivax* malaria. It is known that the expression of DARC and susceptibility to *P. vivax* malaria is influenced by the presence of specific DARC genotypes. Studies have reported the low probability of *P. vivax* malaria in individuals carrying FyA allele due to the significantly reduced binding of the *P. vivax* duffy binding protein. No association of the allele frequency and severe vivax malaria epidemiology has been yet established in our country. In the present study, a high level of heterozygotes was observed with a statistically significant deviation from the H-W equilibrium in the group with complicated malaria; which is indicative of demographic disequilibrium. Significantly upregulated expression of the DARC receptor in FyA/FyB heterozygote patients is suggestive of a greater receptor repertoire responsible for the possible variation in the parasite ligand binding with the host receptor and thus might have a role to play in severe malaria.

Molecular interactions between the host RBCs and the invading parasitic merozoite is the mainstay of the multistep invasion mechanism [1]. DARC is a single copy gene which encodes Duffy antigen consisting of two exons. DARC is a glycosylated transmembrane protein comprising of an extracellular N-terminal domain, seven transmembrane domains and a short cytosolic C-terminal domain [2]. DARC antigen could be recognized by the three different alleles at the genetic level; FY*A, FY*B and FY*O, where FyA and FyB can be differentiated by a single G125A point mutation (Gly 42 Asp), whereas the FyO allele is generated due to the T to C substitution in gene's promoter region at nucleotide position-33 leading to complete absence of the DARC on RBCs [3,4]. Recent studies have reported the low probability of *P. vivax* malaria in individuals carrying FyA allele due to the significantly reduced binding of the *P. vivax* duffy binding protein [5]. Cavisini et al. have suggested that quantitative and/or qualitative variations affecting the Duffy antigen expression on erythrocytes might be responsible for modulating the susceptibility to *P. vivax* malaria in heterozygote individuals [6]. Assessing the DARC expression, its genotype and severe *P. vivax* malaria epidemiology will help in establishing the link between susceptibility towards vivax malaria and genetic structure of DARC.

The present study was approved by the Institute's Ethics Committee (Reference number: Histo/14/2926). The study was carried out on the *Plasmodium vivax* infected patients (collected from PGIMER, Chandigarh) and 70 heathy individuals (afebrile at the time of sample collection with no history of malaria also negative for *P. vivax* by multiplex nested PCR). The *P. vivax* positive samples were further divided into complicated and uncomplicated group on the basis of WHO criteria [8]. The 1096 bp region of Fy/Duffy antigen receptor for chemokine (DARC) gene was targeted using the two primer pairs ((FyAB (exon-2) and FyO (promoter)) as described earlier by Chittoria et al. [9]. One sample from Cameroonian individual (worked as colleague) was taken as a positive control for the sequencing of promoter region of DARC gene. Amplified products were purified using the Gel extraction kit (Qiagen, Germany) as per manufacturer's instructions. The purified products were then subjected to Sanger sequencing as per manufacturer's instruction (ABI, USA) in both forward and reverse directions using respective PCR primers. The DNA sequence chromatograms of FyAB and FyO genes were visually inspected for the presence of single or double peak at the T-33C and G125A nucleotide positions for all *P. vivax* patients and control samples [9]. The statistical analyses and the Hardy-Weinberg equilibrium analysis was conducted as previously described [10]. The expression of DARC was studied in both complicated (n = 15) and uncomplicated (n = 19) group of patients using flowcytometry as described in the Supplementary File 1. To study the expression of DARC on the surface of RBCs; monoclonal mouse anti DARC antibody (Fy6 antibody) labelled with phycoerythrin (PE) (Abcam, Cambridge) were used. To identify the differential expression of DARC receptor on RBCs the non-parametric Mann-Whitney *U* test was applied

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Table 1
Hardy Weinberg expectations, genotype and allele frequency of Fy gene in *P. vivax* infected and control group.

	Observed Genotypes				Allele frequency	
	FYA/FYA n (%)	FYA/FYB n (%)	FYB/FYB n (%)	HWE (χ2)	FYA (%)	FYB (%)
Combined (n = 130/143)	44 (33.8)	76 (58.5)	10 (7.7)	8.46**	63	37
Complicated (n = 48/51)	16 (33.3)	30 (62.5)	2 (4.2)	6.44*	65	35
Uncomplicated(n = 82/92)	28 (34.1)	46 (56.1)	8 (9.8)	3.05	62	38
Control (n = 70/70)	23 (32.9)	36 (51.4)	11 (15.7)	0.25	59	41

HWE- Hardy Weinberg expectations.
* $p < .05$.
** $p < .005$.

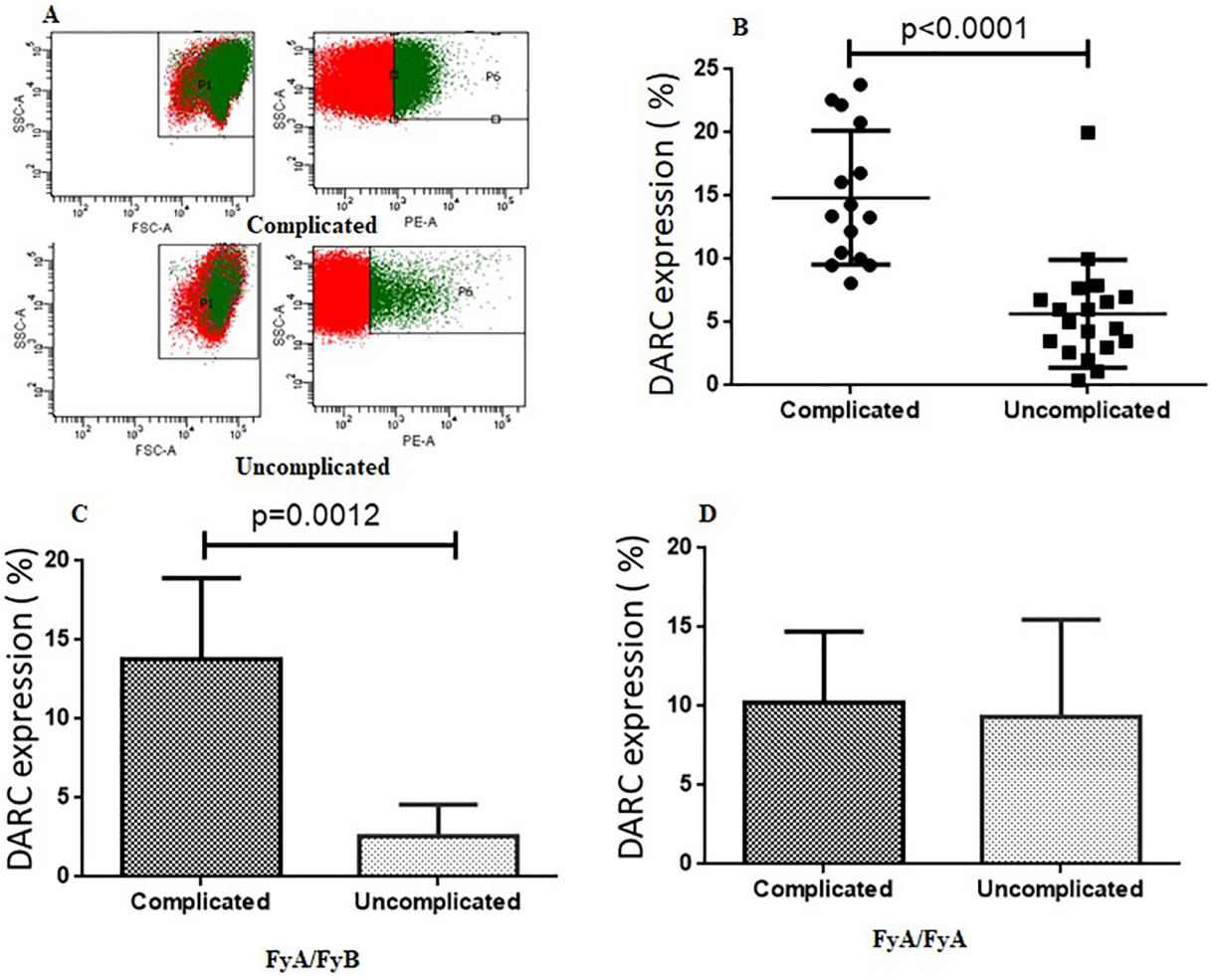

Fig. 1. Analysis of expression of DARC: A) Representative flow cytometric analysis of PE labelled Fy6 antibody specific fluorescence intensity among erythrocytes of complicated and uncomplicated group of patients B) Comparative expression of DARC between complicated & uncomplicated *P. vivax* patients C, D) Comparative expression of DARC between different genotypes (FyA/FyB and FyA/FyA) of uncomplicated & complicated *P. vivax* patients.

and $p < .05$ was considered statistically significant. Statistical analysis was carried out using Graph Pad prism. Version 5.01.

The sequencing of the exon-2 region (FyAB) and promoter region (FyO) was successful in a total of 90.9% (130/143) patients and 100% (70/70) control samples. Among the sequenced *P. vivax* positive and control samples, all of them (100%) were found to be Duffy positive with the presence of T at 33rd nucleotide position in the promoter region. The presence of C nucleotide in a single peak at nucleotide position 33rd indicates the absence of DARC in the positive control. The sequence analysis of exon-2 region reveals the presence of G and A nucleotide at 125th nucleotide position in the *P. vivax* patient and control population samples. These were further characterized into three different genotypes; two homozygotes (FYA/FYA and FYB/FYB) and one heterozygote (FYA/FYB). The majority of the individuals were heterozygotes (FyA/FyB) in both the complicated (62.5%; 30/48) and uncomplicated (56.1%; 46/82) group of *P. vivax* patients and control group (51.4%; 36/70). The homozygote FyA/FyA was found to be the second highest in all three groups of individuals with the frequency of 33.3% (16/48), 34.1% (28/82) and 32.9% (23/70) in complicated, uncomplicated and control group respectively. The highest allelic frequency was observed for FyA allele in all the three groups as compared to the FyB allele. The Hardy Weinberg expectations, frequency distribution of all the genotypes (FyA/FyB, FyA/FyA and FyB/FyB) with allele frequency in *P. vivax* patients and control individuals are shown

in Table 1. However, a significant departure from HW equilibrium in DARC gene was observed in the group with complicated malaria as compared to the group with uncomplicated malaria.

The other SNPs found in the patient and control individuals were present at position 298 in exon-2 (G298A) of the DARC gene. The majority of the complicated 72.9% (35/48) and uncomplicated 75.6% (62/82) *P. vivax* patients and control 77.1% (54/70) individuals were found to have GG homozygotes, AG heterozygous combinations were seen in 25% (12/48) in complicated, 23.2% (19/82) in uncomplicated and 22.9% (16/70) in control population. Whereas only 1.6% (2 patients), of the patients was found to carry the mutant AA genotype at G298A. All the FyA/FyA genotype of DARC were found to have the GG genotype at G298A position, whereas the FyA/FyB and FyB/FyB genotype were found to possess the AG, AA and GG genotypes at G298A position. The expression of the DARC receptor was found to be statistically significant upregulated in complicated group (14.14 ± 4.93 (% ± SD)) as compared to the uncomplicated group (6.85 ± 4.77 (% ± SD)) of patients ($P < .001$). Also, a statistically significant upregulation (Mann–Whitney U, $p < .0012$) of DARC expression was found to be associated with the presence of FyA/FyB among complicated group (13.7 ± 5.15 ((% ± SD)) of patients as compared to uncomplicated group (2.6 ± 1.9 ((% ± SD)) ($p < .0012$) of patients (Fig. 1). Whereas no significant difference in the DARC expression was observed among the patients of two groups with FyA/FyA genotype.

The present study results are in complete concordance with the previous reports from Latin America, Iran where high frequency of FyA/FyB heterozygote genotype (29–40%) was observed in *P. vivax* patients and in healthy blood donors (22–46.6%) (Without history of malaria) [11–14]. Studies by King et al. have reported the low probability of *P. vivax* malaria in individuals carrying FyA allele [5]. The less susceptibility towards *P.vivax* was also seen among the DARC homozygous FyA/FyA population of North-east India [9]. In accordance with the previous study by Moghaddam et al., the findings from the present study are suggestive of the increased susceptibility of FyA/FyB genotype to *P. vivax* malaria [14]. A statistically significant deviation from the H-W equilibrium was observed in the present study in the complicated group of patients; indicate demographic disequilibrium or the occurrence of outbreeding events in the population. In a previous study by Woolley et al., individuals with FyA/FyB heterozygote combination were found to express higher DARC on the red blood cells, than the FyA/FyA and FyB/FyB homozygote individuals [15]. As a consequence, it might be possible that the FyA/FyB heterozygote individuals have a greater receptor repertoire responsible for the possible variation in the parasite ligand binding with the host receptor [7]. The statistically significant deviation from the Hardy-Weinberg equilibrium in complicated group of patients clearly states the demographic disequiliribium in the malaria suscpected population with altering genotype frequencies of DARC gene. Presence of high level of DARC expression with high level of heterozygosity (FyA/FyB genotype) might have a role to play in the increased susceptibility to *P. vivax* malaria.

Competing interests

The authors have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2019.03.013.

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