Online journal ISSN - 1676-5680
GMR Genetics and Molecular Research
EVOLUTION AND TECHNOLOGY

Genetic polymorphisms in TLR4, CR1 and Duffy genes are not associated with malaria resistance in patients from Baixo Amazonas region, Brazil

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Genet. Mol. Res. 7 (4): 1011-1019 (2008)
Received January 28, 2008
Accepted July 25, 2008
Published October 7, 2008

ABSTRACT. The main purpose of this research was to analyze the relation of the genetic polymorphisms frequently expressed by antigen-presenting cells, erythrocytes and malaria susceptibility/resistance with the human malaria infection cases. The sample used consisted of 23 *Plasmodium vivax* (Pv)- and *P. falciparum* (Pf)-infected patients, and 21 healthy individuals as a control group, from the Baixo Amazonas population in Pará, Brazil. The Asp299Gly polymorphisms in the Toll-like receptor 4 (TLR4), and Gly42Asp, Arg89Cys, Ala100Thr, and T-33C in the Duffy gene (FY) were analyzed by restriction fragment length polymorphism-polymerase chain reaction. The Lys1590Glu and Arg1601Gly polymorphisms in the

complement receptor type 1 (CR1) were analyzed by DNA sequencing. According to the results obtained and statistical analysis considering a significance level or $\alpha = 0.01$, we conclude that the low heterozygote frequency (2.27%) for the Asp299Gly mutation, detected in the TLR4 gene, is not related to the Pv and Pf infections in the patients analyzed. Also, the promoter region GATA-1 analysis of the FY gene in the Pv-infected patients showed that the heterozygote frequency for the T-33C mutation (11.36% of the infected patients and 20.45% of the control patients) is not related to infection resistance. Regarding the CR1 gene, the observed heterozygote frequency (9.09%) for the Arg1601Gly mutation in Pf-infected patients when compared to heterozygote frequency in the control group (18.18%) suggests that there is no correlation with infection resistance.

Key words: Malaria; Complement receptor-1; Toll-like receptor; Duffy blood group; Genetic polymorphisms; Brazilian population

INTRODUCTION

In Brazil, malaria transmission occurs essentially in the Amazon region, where more than 99% of infections are acquired and which is mainly associated with road constructions, production projects and mining area expansion (Marques et al., 1986). The Brazilian Amazon region is propitious for the development of malaria transmitting mosquitoes, where transmission is facilitated by the precarious habitations increasing near the closed plants and backwaters without medical and/or preventive health assistance.

The serious manifestations and clinical spectrum development of malaria can be determined by the few aspect interactions, such as: parasitemia, acquired immunity level, nutritional conditions, age, genetic factors, income level, and parasite characteristics including growth ratio, drug resistance and, perhaps, geographic origin (Greenwood et al., 1991). Several parasite species are known but only four varieties cause human infection. In particular, *Plasmodium falciparum* (Pf) and *Plasmodium vivax* (Pv), in the Brazilian Amazon region (Breman, 2001).

The erythrocyte invasion by the malaria parasite is a complex process involving specific binding-receptor interactions between the blood stage merozoite form and erythrocyte surface receptors (Chitnis, 2001). *P. vivax* and *P. falciparum* make use of a family of binding proteins on the surface of red blood cells (RBCs) defined by the occurrence of a high concentration of cysteine regions (Adams et al., 1992). The Pv invasion of human RBCs depends on the Duffy-binding protein and the erythrocyte Duffy antigen (Fy) interaction (Chitnis, 2001). The Duffy blood group polymorphism negativity is important in areas where Pv predominates (Marques et al., 1986), conferring a natural resistance against parasite infection in individuals from endemic regions due to the absence of Fy (Pogo and Chaudhuri, 2000). Pf uses different binding receptors but in a similar manner compared to the Duffy-binding protein of Pv for erythrocyte invasion (Baum et al., 2003). After the invasion, Pf has the ability to induce the adhesion between infected and non-infected erythrocytes, using the erythrocyte membrane protein-one interaction (PfEMP1) and complement receptor-1 (CR1) of human RBCs, inducing rosette formation (Rowe et al., 1997). This process leads to blood flow obstruction due to peripheral resistance, limits local oxygenation and causes

attachment of RBCs to the vascular endothelium, which gives rise to continuous liver and spleen damage (Miller et al., 2002). This phenomenon, associated with severe malaria cases, plays a direct role in the pathogenicity of infection (Rowe et al., 1995, 2000). Moreover, the individuals who have this kind of polymorphism develop a low expression of CR1 inducing a decrease in rosette formation, making them resistant to the development of severe malaria (Rowe et al., 1997).

Gene analysis combined with innate immunity is the molecular basis of the observed clinical heterogeneities in hosts infected with *Plamodium* sp. Recent studies suggest that the Toll-like receptors (TLRs), mediators of a signaling cascade that involves, especially, recruitment of the adaptor protein, MyD88, lead the proinflammatory cytokine production mainly in antigen-presenting cells. The process involves an immune response against a variety of pathogens, including *Plasmodium* (Adachi et al., 2001; Supajatura et al., 2002; Coban et al., 2005; Krishnegowda et al., 2005; Perry et al., 2005). Proinflammatory cytokines such as TNF-α, interferon-γ, IL-12, IL-1, and IL-6, and nitric oxide, produced during malaria infection, are critical to control parasite growth (Balmer et al., 2000). Nowadays, TLR1 to TLR13 have been identified in mammalian cells, most recognizing specific pathogen-associated molecular patterns (Tabeta et al., 2004). TLR polymorphisms are characterized by inducing limited proliferation of phagocyte mononuclear cells, suppressing the innate immune response and increasing the susceptibilities of the host to bacterial infections (Malley et al., 2003). In patients with malaria, the destruction of infected hepatocytes reduces T lymphocyte numbers, leading to the increased parasitemia (Adachi et al., 2001).

Malaria has a significant association with a variety of polymorphisms that occur in RBCs and cells of the natural immunity system detected in populations who live in endemic regions. Specifically, the Amazon region supplies an excellent test source to study these associations, and others such as the associations of the different mechanisms between genetic markers and genes involved with *Plasmodium* sp infection susceptibilities/resistance (Beiguelman et al., 2003). In the present study, the following genes were selected for study: Toll-like receptor (TLR4), Duffy (FY) and complement receptor type 1 (CR1), since association polymorphisms can be related to *P. vivax* and *P. falciparum* susceptibilities/resistance leading to an evaluative selection in malaria endemic regions such as the Brazilian Amazon.

SUBJECTS AND METHODS

Subjects

The sample consisted of 44 volunteers who signed an informed consent form, including 23 patients infected with *P. vivax* (14) and *P. falciparum* (9) and 21 healthy individuals comprising the control group, all living in the Baixo Amazonas region in Pará, Brazil.

Statistical analysis of the data was performed by the chi-square test, considering the significance level of $\alpha = 0.01$.

DNA extraction/polymerase chain reaction

The DNA was extracted using the super Quik gene-DNA isolation kit (AGTC, Denver, CO, USA), according to manufacturer instructions. Polymerase chain reaction amplification was processed in a total volume of 25 μL in the Thermocycler Gene Amp PCR System (Perkin Elmer 9700, Norwalk, CA, USA). The reagents used for amplification were 1.5 mM Tris-HCl,

pH 8.5, 0.1 M KCl, 1.5 mM $MgCl_2$, 1 µg/µL bovine serum albumin, 0.1 µM of each primer (Table 1), 100 mM dNTPs, and 1 unit of Biotools DNA polymerase (B&M Labs, Madrid, Spain). Particularly, the forward primer used for TLR4 sequence reaction was prepared with the cytosine addition in substitution of a thymine in the 3' end region, creating a recognition sequence (C**C**ANNNNN/NTGG) to the *Bst*XI endonuclease restriction, called primer mismatch (Table 1) and reported by Okayama et al. (2002).

Table 1. Primer sequences of the TLR4, FY (Duffy) and CR1 genes.

Primer	Sequence of primer	Temperature
TLR4 F	5' AGC ATA CTT AGA CTA C*CA CCT CGA TG 3'	72°C
TLR4 R	5' GTT GCC ATC CGA AAT TAT AAG AAA AG 3'	94°C
		57°C
GATA F (FY)	5' CAA GGC CAG TGA CCC CCA TA 3'	72°C
GATA R (FY)	5' CAT GGC ACC GTT TGG TTC AG 3'	95°C
		58°C
FY F	5' CCC TCT TGT GTC CCT CCC TTT 3'	72°C
FY R	5' CAG AGC TGC GAG TGC TAC CTA 3'	95°C
		58°C
CR1 F	5' TAA AAA ATA AGC TGT TTT ACC ATA CTC 3'	94°C
CR1 R	5' CCC TCA CAC CCA GCA AAG TC 3'	60°C
		72°C

Primer F = forward; Primer R = reverse. *C = position where a cytosine was added to generate a primer mismatch.

Restriction fragment length polymorphism determination

The Asp299Gly polymorphism determination in the TLR4 (A896G) genes followed the protocol suggested by Okayama et al. (2002). The determination of the Gly42Asp (G125A), Arg89Cys (C265T), Ala100Thr (G298A) polymorphisms of the Duffy gene (FY) coding region followed the protocol suggested by Parasol et al. (1998), and the T33C polymorphism determination of the Duffy gene (FY) promoter region (GATA-1 box) followed the protocol suggested by Yazdanbakhsh et al. (2000). For the above polymorphisms the following restriction enzymes were used: *Bst*XI, *Ban*I, *Acil*, *Mwo*I (New England Biolabs, MA, USA) and *Sty*I (Gibco BRL, MD, USA), respectively, and the fragments were analyzed on 8% polyacrylamide gel (Figure 1 and Figure 2A, B, C, respectively). The reaction conditions used were: a) 4 h at 37°C for *Ban*I, *Acil* and *Sty*I, b) 4 h at 60°C for *Mwo*I, and c) overnight at 55°C for *Bst*XI.

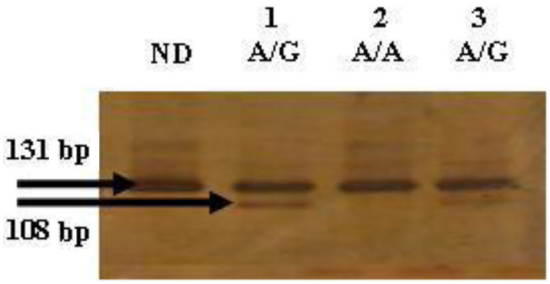

Figure 1. The Asp299Gly restriction fragment length polymorphism. ND = PCR product not digested. Lanes 1, 2, 3 = PCR product after *Bst*XI digestion.

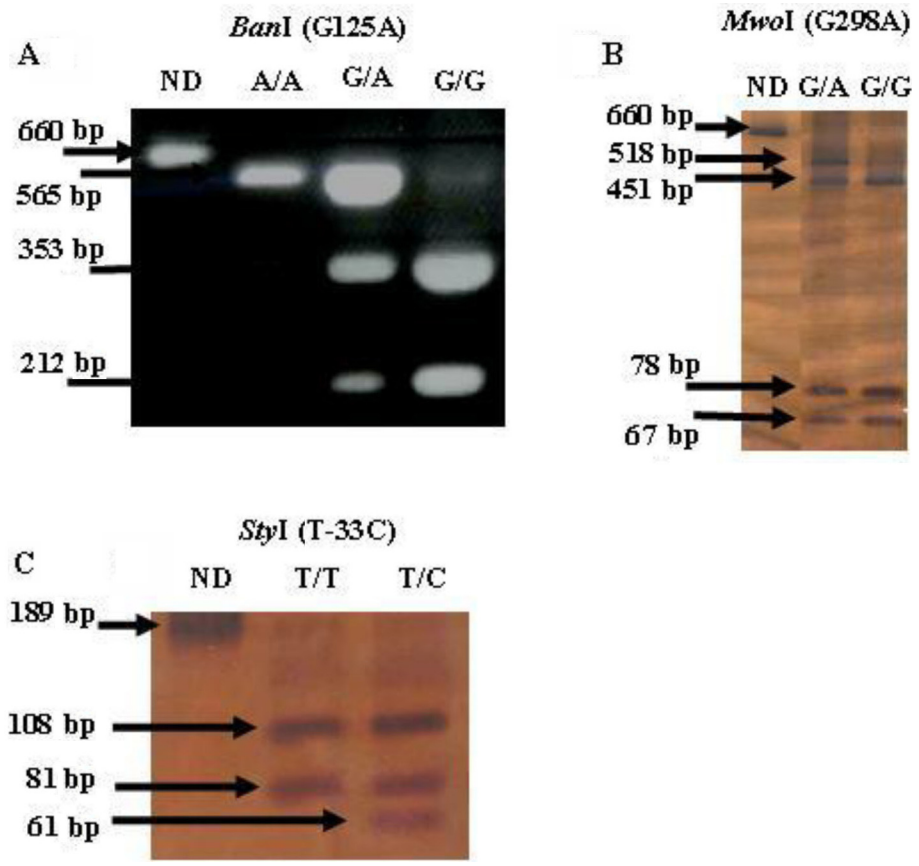

Figure 2. A. The Gly42Asp; **B.** Ala100Thr, and **C.** T-33C restriction fragment length polymorphism. ND = PCR product not digested. Lanes 1, 2, 3 = PCR product after *Ban*I, *Mwo*I and *Sty*I digestion, respectively.

DNA sequencing

The Lys1590Glu and Arg1601Gly polymorphisms identified by Moulds et al. (2001) in the CR1 gene were sequenced using fluorescence-based sequencing protocols in an ABI377 automated sequencer (Perkin Elmer, USA).

RESULTS AND DISCUSSION

The Asp299Gly polymorphism of the TLR4 gene has been associated with the reduction in receptor signaling and, as a consequence, it reduces inflammatory response (Adachi et al., 2001; Guo et al., 2005; Hellmig et al., 2005; Minoretti et al., 2006). Adachi et al. (2001) associated the increase in parasitemia level in *P. berghei*-infected mice with mutations in the Toll-like receptors. Based on this information, we investigated if *P. vivax* and *P. falciparum*

have the same relation with infection susceptibilities in patients diagnosed with malaria from the Baixo Amazonas/Pará region.

According to the results obtained and shown in Table 2, it can be seen that only one patient (2.27%) and four controls (9.09%) have the Asp299Gly mutation in heterozygous, corresponding to $\chi^2 = 2.35$ (P = 0.125). These data indicate no relation between the mutation in heterozygosis with the *P. vivax* and *P. falciparum* susceptibility infections due the low frequency in the patients. On the other hand, the control group showed a higher superior mutation frequency.

Table 2. The Asp299Gly mutation frequency.

	Infected	Non-infected	Total
With mutation	1 (2.27%)	4 (9.09%)	5 (11.36%)
Without mutation	22 (50%)	17 (38.63%)	39 (88.63%)
Total	23 (52.27%)	21 (47.72%)	44 (100%)

Data are reported as number with percent in parentheses.

The differences in the Duffy phenotype frequency in the ethnic groups and distinct geographic populations have been attributed to the positive selection of individuals with phenotype Fy(a-b-), characterized by being resistant to the *P. vivax* infection (Chown et al., 1965; Lewis et al., 1972; Daniels, 1997). Among the individuals analyzed, the Arg89Cys mutation related with the FY^b (Fy^x) weakened phenotype was not found. However, frequency variability was observed in the Ala100Thr silent mutation (Figure 2B), T-33C (Figure 2C) that cause the deficiency of gene FY and the Gly42Asp mutation (Figure 2A), responsible for FY*A/FY*B phenotype occurrence. Table 3 compares the results of Duffy genotyping and phenotyping, as well as Fy allele frequency, in *P. vivax*-infected patients and non-*vivax* controls. All individuals were heterozygote for the mutations.

Table 3. The phenotype, genotype and allelic frequency related to Duffy's blood group.

GATA-1	Genotype	Deduced phenotype	Frequency	
			Patients[a]	Controls[b]
wt/wt	Fy*A/Fy*A	Fy(a+b-)	14% (2/14)	30% (9/30)
wt/m	$Fy*A/Fy*B^{ES}$	Fy(a+b-)	28% (4/14)	26% (8/30)
wt/wt	Fy*A/Fy*B	Fy(a+b+)	50% (7/14)	30% (9/30)
wt/wt	Fy*B/Fy*B	Fy(a-b+)	-	6% (2/30)
wt/m	$Fy*B/Fy*B^{ES}$	Fy(a-b+)	7% (1/14)	6% (2/30)

[a]Patients infected with *P. vivax*. [b]Healthy controls and patients infected with *P. falciparum*. wt = wild-type; m = mutated (i.e., showing the T-33C substitution).

The results obtained (Table 3) demonstrate the Fy(a-b-) phenotype absence and the Fy(a+b-) and Fy(a-b+) phenotype presence, related with the $Fy*A/Fy*B^{ES}$ and $Fy*B/Fy*B^{ES}$ genotypes in the samples analyzed, respectively. These results characterize the FY gene partial deficiency, being heterozygote for T-33C mutation.

Table 4 shows that only five patients (11.36%) and nine controls (20.45%) had the T-33C mutation in heterozygous, corresponding to $\chi^2 = 0.14$ (P = 0.7047). This analysis is corroborated by Cavasini et al. (2001) who established a non-correlation of the *P. vivax* in-

fection resistance in heterozygote for T-33C mutation due to the low frequency in patients. Moreover, the results obtained confirmed the findings of Estalote et al. (2005) who showed that Ala100Thr silent mutation can occur not only in Caucasians but in other populations and also does not affect Duffy expression on red blood cells.

Table 4. The T33C mutation frequency.

	Infected	Non-infected	Total
With mutation	5 (11.36%)	9 (20.45%)	14 (31.81%)
Without mutation	9 (20.45%)	21 (47.72%)	30 (68.18%)
Total	14 (31.81%)	30 (68.18%)	44 (100%)

Data are reported as number with percent in parentheses.

The lines of evidence analyzed suggest that polymorphism involvement associated with the CR1 gene led to better survival advantages in patients in malaria endemic regions (Rowe et al., 1995, 1997; Moulds et al., 2000, 2001; Miller et al., 2002). In this case, *P. falciparum*-infected patient DNA was compared with that of non-*falciparum* controls.

The observed allelic frequency for Lys1590Glu and Arg1601Gly mutations (Tables 5 and 6), in *P. falciparum*-infected patients when compared with the control group does not suggest that heterozygosis can influence the infection resistance for this *Plasmodium* species due to the low frequency in the patients. According to the results obtained and shown in Table 6, it can be demonstrated that only four patients (9.09%) and eight controls (18.18%) had the Arg1601Gly mutation in heterozygous, corresponding to $\chi^2 = 1.68$ (P = 0.1947).

Table 5. The Lys1590Glu mutation frequency.

	Infected	Non-infected	Total
With mutation	1 (2.27%)	1 (2.27%)	2 (4.54%)
Without mutation	8 (18.18%)	34 (77.27%)	42 (95.45%)
Total	9 (20.45%)	35 (79.54%)	44 (100%)

Data are reported as number with percent in parentheses.

Table 6. The Arg1601Gly mutation frequency.

	Infected	Non-infected	Total
With mutation	4 (9.09%)	8 (18.18%)	12 (27.27%)
Without mutation	5 (11.36%)	27 (61.36%)	32 (72.72%)
Total	9 (20.45%)	35 (79.54%)	44 (100%)

Data are reported as number with percent in parentheses.

The high Arg1601Gly mutation frequency in heterozygosis in the samples is understandable, since two variants studied are responsible for the McC (b+) and Sl (-) phenotypes of the Knops blood group system, which is more frequent in African populations than in Caucasians (Rowe et al., 1995; Moulds et al., 2000). This indicates that the Baixo Amazonas/Pará population has intense African miscegenation as evidenced in the Duffy blood group system analysis.

It can also be concluded that there was no correlation with parasitemia level (high variability) when compared to the heterozygotic frequency observed for the TLR4, FY and CR1 genes.

ACKNOWLEDGMENTS

We thank Ane Mari Dinarte Santos and Cristiane Ayres Ferreira for technical assistance and José Barbosa Filho for statistical analysis. Research supported by Fundação Hemocentro de Ribeirão Preto, São Paulo, and CAPES.

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