Genetic polymorphism of *Plasmodium vivax* Duffy Binding Protein in malarious areas in southeastern of Iran

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Received: 18 March 2017 / Accepted: 15 July 2017 / Published online: 20 July 2017
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Abstract *Plasmodium vivax* parasite causes the largest number of malaria infection in some malarious areas of the world including Iran. Considering transfer and genetic dynamics of the parasite population in a specific area can help us to predict the spread of the infection either emergence of new cases or drug resistance in the context of elimination program in the malarious areas. Study on the genetic diversity of common alleles in a given geographical area, for vaccine and immune level studies can be important. The purpose of this study was to know the status of *P. vivax* Duffy Binding Protein (PvDBP) polymorphism in patients infected with the parasite in malaria endemic southeastern Iran. The fragment of gene corresponding to PvDBP of thirty *P. vivax* malaria infected individuals was amplified. A 1176 bp band related to this fragment was purified and PCR–RFLP method was employed using enzymatic digestion with PstI and RsaI restriction enzymes. Ten percent of samples were sent for sequencing. PCR–RFLP showed that 99.7% of the samples were cut as the same together, either the PstI enzyme or the enzyme of RsaI. In each case, only 2 isolates were unlike others. Findings revealed that there is at least 96% identity among isolates in the nucleotide level. Amino acid pattern of PvDBP in Iranian isolates showed little discrepancies with those PvDBP genes that have been recorded in GenBank.

Sequencing of PvDBP isolates of Iranian *P. vivax* infected patients showed low level of genetic polymorphism among them. Results of this study can prepare valuable information for malaria policy makers to intend them in their malaria control program.

Keywords *Plasmodium vivax* · Duffy Binding Protein · Restriction enzymes · Iran

Introduction

Malaria is one of the most public health threatening parasitic diseases in the world. According to the World Health Organization (WHO) report the number of malaria cases was 214 million annually (WHO 2015). Among the malaria infected cases, *Plasmodium vivax* is responsible for the largest number of the disease in some malarious areas including some Middle East countries (WHO 2011a). Although *vivax* malaria is not as malignant as falciparum malaria, some facts such as relapses, malignancy in splenectomy individuals make the disease a debilitating illness (Antinori et al. 2012). In despite of above mentioned complications some individuals rarely catch the disease due to inherent facts mainly lacking Duffy antigen receptor for chemokines (DARC) expression.

A number of proteins on the surface of human erythrocytes play important roles in invasion of human *Plasmodium* merozoites to bind to the erythrocyte (Cowman and Crabb 2006). *Plasmodium vivax* Duffy Binding Protein (PvDBP) plays an essential role in erythrocyte invasion and supposed to be a potential asexual blood stage vaccine candidate antigen against *P. vivax*. Dependence of *P. vivax* to Duffy binding protein during invasion of erythrocytes is a crucial step of junction formation. Such process provides

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a unique opportunity for development of an effective intervention strategy against *P. vivax* infections. Since strong immune responses to PvDBP in human, the protein is seemed to be an important asexual blood stage vaccine candidate against *P. vivax* (Michon et al. 1998; Xainli et al. 2002). According to some experimental studies antibodies against PvDBP can block the invasion of the parasite into erythrocytes (Michon et al. 2000). *Plasmodium vivax* DBP is 140-kDa microneme-secreted protein with crucial role that participates in merozoite invasion to human Duffy antigen positive erythrocytes (Grimberg et al. 2007; Wertheimer and Barnwell 1989; Miller et al. 1976). The protein is characterized by functionally conserved amino terminal cysteine-rich region (VanBuskirk et al. 2004) that contains the binding motifs necessary for adherence to Duffy antigen receptor for chemokines (DARC) on the erythrocyte (Cole-Tobian et al. 2002). The main binding motifs in *P. vivax* DBP II have been placed in a region between amino acids 291 and 460 including 5–8 cysteines (Chitnis and Miller 1994; Chitnis et al. 1996). Genetic variation analysis of PvDBP regions among some isolates of *P. vivax* in different geographical areas indicated that PvDBP II shows the highest genetic diversity which leads to a highly polymorphic component in the regions (King et al. 2002; Xainli et al. 2000; Fraser et al. 1997; Michon et al. 1998). Although such polymorphism may alter some targets in immune recognition of PvDBP (King et al. 1991), a number of studies are on the contrary to the above mentioned suggestion indicating that alters do not affect host-parasite binding in significant level (Rudensky et al. 1991; Fraser et al. 1997). Indeed, understanding the genetic polymorphism and nature of DBP in *P. vivax* isolates in various malaria endemic areas especially where a large proportion of malaria infection belongs to *vivax* malaria is an important and rational action. In this study we tried to determine the genetic polymorphism of PvDBP in field isolates using PCR–RFLP method collected from malarious areas of southeastern Iran.

Materials and methods

Study areas and blood samples

Thirty blood samples were collected from Hormozgan and Sistan and Baluchistan provinces. Both provinces are malaria endemic areas located in southeastern Iran possess warm weather with average temperature of 36 °C in spring and summer and 25 °C in autumn and winter. *P. vivax* infected samples were confirmed using malaria microscopy technique based on WHO recommendation (2011b). Two milliliter blood sample was collected from each of the enrolled individuals and deposited into EDTA

contained tubes for extracting DNA of the parasite and detecting PvDBP. Previous to blood sampling an informed consent was obtained from all of the participated individuals based on permission and regulation of Tehran University of Medical Sciences Ethics Committee. The prepared samples were stored at –20 °C until PCR processes.

DNA extraction and PCR amplification

Genomic DNA was extracted from whole blood collected of patients using DNA Extraction kit (Genomic DNA Extraction Kit, Korea Bioneer AccuPrep), based on manufacture instructions.

Polymerase chain reaction (PCR) was performed for PvDBP gene. Amplification was done using a set of following primers which were designed according to the sequence of *P. vivax* Sal-1 isolate submitted in GenBank (accession NO: XM-001615397.1) for amplification of PvDBP gene.

PvF: 5'GAT TAT GAG ACA TCT AGC AAT GG 3'

PvR: 5'ACT ATC TAC AGG CTG ACT TAT CG 3'

PCR was performed in a total volume of 25 µl. The solution was composed of 10 pmol of each primer concentrations, 20 ng/µl template and amplicon Master Mix (comprising 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.025 u/µl amplicon taq DNA polymerase 75 mM Tris HCl, 20 mM (NH₄)₂ SO₄). PCR assay for amplifying included the following schedule: initial denaturation in 94 °C for 5 min followed by 35 cycle of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, extension in 75 °C for 1 min and the final extension for 10 min. Electrophoresis was employed for PCR products, then the desired band with 1176 bp was extracted from gel agarose using QIA quick Gel extraction Kit (Cat. No. 28704) based on guidelines of the company. PCR–RFLP was carried out for the PCR productions using RsaI and PstI restriction enzymes and finally 13.3% of samples (PCR products) were sent to Takapouzist Company (Tehran, Iran) for sequencing process. Nucleotide and amino acid sequence alignment were performed using ClustalW2 and ExPASy softwares.

Results

As it has been shown in Table 1 a total of 30 samples were collected in this study. Regarding to nationality 63.3% of them were Iranian people, 33.4 and 3.3% were Afghan and Pakistani individuals respectively. Most of the population was in group of 20–40 years old, 6.7% were over than that and 40% were under 20 years old. In terms of sexuality, most of them (70%) were men.

Table 1 Distribution of population including nationality, age and gender

Characteristics	Nationality			Age			Gender		Total
	Iranian	Afghan	Pakistan	<20	20–40	>40	Female	Male	
No. of patients	19	10	1	12	16	2	9	21	30
Percentage	63.3%	33.4%	3.3%	40%	53.3%	6.7%	30%	70%	100%

Fig. 1 Gel electrophoresis of PCR products corresponding to PvDBP. SM: DNA size marker, columns 1–8: a 1176 bp band matching with PvDBP of samples, column 9: negative control **Fig. 2** Agarose gel electrophoresis of PvDBP gene affected by PstI enzyme. Columns 11–14 were related to samples digested by the restriction enzyme and produced two fragments (387 and 789 bp), but isolate No. 15 was not cut (accession number: KF751809.1)

A genomic fragment of DNA corresponding to PvDBP with a weight about 1176 bp was yielded from all of the 30 collected samples, selected piece was similar with other data stored in GenBank (Fig. 1).

Two fragments 387 and 789 bp were produced after affecting *Plasmodium vivax* Duffy protein gene by PstI restriction enzyme (Fig. 2). The gene also affected by the RsaI restriction enzyme which resulted to three pieces including 199, 346 and 631 bp (Fig. 3).

Four samples out of 30 samples were sequenced after the PCR–RFLP processing. Finally, all four isolates were registered in GenBank as the bah1, bah2, bah3, bah4 isolates (accession numbers: KF751807.1, KF751808.1, KF751809.1, KF751810.1 respectively), then compared with those isolates reported from India (accession number: DQ156514), Korea (SK1, SK2 isolates with accession numbers AF215738.1, AF215737.1 respectively) and

Fig. 3 Gel electrophoresis of PCR–RFLP productions using RsaI restriction endonuclease on PvDBP gene. SM: DNA size marker. Columns 11–18 are related to enzymatic digestion of the samples, Column 19 is negative control. As it is seen in the figure, sample No. 18 wasn't cut by the RsaI restriction enzyme while the rest of the samples were cut into three fragments including of 346 and 631 and 199 bp

bah4	RKNSSNGGNPYDIDHKKTISSAIINHAFQNTVMKNCNYKRRRERDWCNTKKDVCIPD	74
bah1	RKNSSNGGNPYDIDHKKTISSAIINHAFQNTVMKNCNYKRRRERDWCNTKKDVCIPD	83
bah2	RKNSSNGGNPYDIDHKKTISSAIINHAFQNTVMKNCNYKRRRERDWCNTKKDVCIPD	72
iran	RKNSSNGGNPYDIDHKKTISSAIINHAFQNTVMKNCNYKRRRERDWCNTKKDVCIPD	88
bah3	RKNSSNGGNPYDIDHKKTISSAIINHAFQNTVMKNCNYKRRRERDWCNTKKDVCIPD	80
india	RKNSSNGGNPYDIDHKKTISSAIINHAFQNTVMKNCNYKRRRERDWCNTKKDVCIPD	240
Sal-1	RKNSSNGGNPYDIDHKKTISSAIINHAFQNTVMKNCNYKRRRERDWCNTKKDVCIPD	240
Sk1	-----ERDWCNTKKDVCIPD	16
sk2	-----ERDWCNTKKDVCIPD	16

bah4	RRYQLCMKELTNLVNNTDTNFHRDITFRKLYLKRKLIYDAAVEGDLKLNRYRNKDFC	134
bah1	RRYQLCMKELTNLVNNTDTNFHRDITFRKLYLKRKLIYDAAVEGDLKLNRYRNKDFC	143
bah2	RRYQLCMKELTNLVNNTDTNLHSDITFRKLYLKRKLIYDAAVEGDLKLNRYRNKDFC	132
iran	RRYQLCMKELTNLVNNTDTNFHSDITFRKLYLKRKLIYDAAVEGDLKLNRYRNKDFC	148
bah3	RRYQLCMKELTNLVNNTDTNFHRDITFRKLYLKRKLIYDAAVEGDLKLNRYRNKDFC	140
india	RRYQLCMKELTNLVNNTDTNFHRDITFRKLYLKRKLIYDAAVEGDLKLNRYRNKDFC	300
Sal-1	RRYQLCMKELTNLVNNTDTNFHRDITFRKLYLKRKLIYDAAVEGDLKLNRYRNKDFC	300
Sk1	RRYQLCMKELTNLVNNTDTNFHRDITFRKLYLKRKLIYDAAVEGDLKLNRYRNKDFC	76
sk2	RRYQLCMKELTNLVNNTDTNFHRDITFRKLYLKRKLIYDAAVEGDLKLNRYRNKDFC	76
	*****;*	
bah4	KDIRWSLGDFGDIIMGTDMEGIGYSEVENNLRISFGTGEKAQQRRKQWWNESKAQIWA	194
bah1	KDIRWSLGDFGDIIMGTDMEGIGYSEVENNLRISFGTGEKAQQRRKQWWNESKAQIWA	203
bah2	KDIRWSLGDFGDIIMGTDMEGIGYSKVVENNLRISFGTGKNAQQHRKQWWNETKAQIWA	192
iran	KDIRWSLGDFGDIIMGTDMEGIGYSKVVENNLRISFGTGKNAQQHRKQWWNETKAQIWA	208
bah3	KDIRWSLGDFGDIIMGTDMEGIGYSKVVENNLRISFGTGEKAQQHRKQWWNESKAQIWA	200
india	KDIRWSLGDFGDIIMGTDMEGIGYSKVVENNLRISFGTDEKAQQRRKQWWNESKAQIWA	360
Sal-1	KDIRWSLGDFGDIIMGTDMEGIGYSKVVENNLRISFGTDEKAQQRRKQWWNESKAQIWA	360
Sk1	KDIRWSLGDFGDIIMGTDMEGIGYSKVVENNLRISFGTDEKAQQRRKQWWNESKAQIWA	136
sk2	KDIRWSLGDFGDIIMGTDMEGIGYSEVENNLRISFGTGEKAQQHRKQWWNESKAQIWA	136
	*****;*****;*****;*****;*****	
bah4	MMYSVKKRLKGKFMWICKINAVNIEPQIYRRIREWGRDYVSELPTEVQKLKEKCDGKIN	254
bah1	MMYSVKKRLKGKFIWICKINAVNIEPQIYRRIREWGRDYVSELPTEVQKLKEKCDGKIN	263
bah2	MMYSVKKRLKGNFIWICKINAVNIEPQIYRWIREWGRDYVSELPTEVQKLKEKCDGKIN	252
iran	MMYSVKKRLKGNFIWICKINAVNIEPQIYRWIREWGRDYVSELPTEVQKLKEKCDGKIN	268
bah3	MMYSVKKRLKGNFIWICKINAVNIEPQIYRWIREWGRDYVSELPTEVQKLKEKCDGKIN	260
india	MMYSVKKRLKGNFIWICKINAVNIEPQIYRWIREWGRDYVSELPTEVQKLKEKCDGKIN	420
Sal-1	MMYSVKKRLKGNFIWICKINAVNIEPQIYRWIREWGRDYVSELPTEVQKLKEKCDGKIN	420
Sk1	MMYSVKKRLKGKFIWICKINAVNIEPQIYRRIREWGRDYVSELPTEVQKLKEKCDGKIN	196
sk2	MMYSVKKRLKGKFIWICKINAVNIEPQIYRRIREWGRDYVSELPTEVQKLKEKCDGKIN	196
	*****;*:*****;*****;*****;*****	
bah4	YTDKKVCKV-PPCQNACKSYDQWITRKKNQWDVLSNKFISVKNAEKVQTAGIVTPYDILK	313
bah1	YTDKKVCKV-PPCQNACKSYDQWITRKKNQWDVLSNKFISVKNAEKVQTAGIVTPYDILK	322
bah2	YTDKKVCKVLPCCQNACKSYDQWITRKKNQWDVLSNKFISVKNAEKVQTAGIVTPYDILK	312
iran	YTDKKVCKVLPCCQNACKSYDQWITRKKNQWDVLSNKFISVKNAEKVQTAGIVTPYDILK	328
bah3	YTDKKVCKV-PPCQNACKSYDQWITRKKNQWDVLSNKFISVKNAEKVQTAGIVTPYDILK	319
india	YTDKKVCKV-PPCQNACKSYDQWITRKKNQWDVLSNKFISVKNAEKVQTAGIVTPYDILK	479
Sal-1	YTDKKVCKV-PPCQNACKSYDQWITRKKNQWDVLSNKFISVKNAEKVQTAGIVTPYDILK	479
Sk1	YTDKKVCKV-PPCQNACKSYDQWITRKKNQWDVLSNKFISVKNAEKVQTAGIVTPYDILK	255
sk2	YTDKKVCKV-PPCQNACKSYDQWITRKKNQWDVLSNKFISVKNAEKVQTAGIVTPYDILK	255
	***** *****	
bah4	QELDEFNEVAFENEINKRDGAYIELCVCSVEEAKKNTQEVVTNVDNAAKSQAT-----	366
bah1	QELDEFNEVAFENEINKRDGAYIELCVCSVEEAKKNTQEVVTNVDNAAKSQATNSNPISQ	382
bah2	QELDEFNEVAFENEINKRDGAYIELCVCSVEEAKKNTQEVVTNVDNAAKSQ-----	363
iran	QELDEFNEVAFENEINKRDGAYIELCVCSVEEAKKNTQEVVTNVDNAAKSQATNSNPISQ	388
bah3	QELDEFNEVAFENEINKRDGAYIELCVCSVEEAKKNTQEVVTNVDNAAKSQATNSN----	375
india	QELDEFNEVAFENEINKRDGAYIELCVCSVEEAKKNTQEVVTNVDNAAKSQATNSNPISQ	539
Sal-1	QELDEFNEVAFENEINKRDGAYIELCVCSVEEAKKNTQEVVTNVDNAAKSQATNSNPISQ	539
Sk1	QELDEFNEVAFENEINKRDGAYIELCVCSVEEAKKNTQEVVTNVDNAAKSQATNSNPISQ	315
sk2	QELDEFNEVAFENEINKRDGAYIELCVCSVEEAKKNTQEVVTNVDNAAKSQATNSNPISQ	315

Fig. 4 CLUSTAL 2.1 multiple sequence alignment (protein)

Salvador-1 (accession number: DQ156512.1). These isolates showed few single polymorphisms in nucleotide sequence in comparison with Sal1, India and Korean isolates.

bah3 isolate had the greatest (99.82%) homology with Sal1 isolate. Also this isolate showed 99.82 and 82.5% homologies with isolates of India and Korea respectively. The percentage of homology between bah4 isolate and India (03-1) and El Salvador isolates was 99.36%. Moreover this isolate showed 83.64% homology with SK1, SK2 isolates from Korea.

bah1 isolate displayed 99.48% homology with El Salvador and India isolates and 82.17% homology with Korean isolates.

The percentage of homology of bah2 isolate was 98.81% by El Salvador isolate. It would be noted that among the four Iranian sequenced isolates the lowest percentage of similarity was determined between bah3 and SK1 isolate with 82.42% identity.

Proteins predicted from the gene sequences submitted in GenBank was obtained using ExPASy software and compared with Sal1 isolate. Results showed that in this study there were eight amino acid substitutions at 261, 263, 326, 372, 374, 379, 391, 459 positions in comparison with sal1 isolate, also in bah2 isolate there is a Lysine amino acid added to the sequence in 430th position. Protein alignment using clustalW2 revealed that in these positions Leucine, Serine, Glutamic acid, Lysine, Methionine, Isoleucine, Arginine, and Lysine are substituted by: Phenylalanine, Arginine, Lysine, Asparagine, Isoleucine, Leucine, Tryptophan, and Isoleucine respectively (Fig. 4).

Discussion

PvDBP contains a vital domain to connect and enter the host cells, so plays an important role in parasite invasion to host cells. The gene expresses a protein with 140 KD weight which takes place in microneme of merozoites. The relevant gene contains some diversities depending on different geographical regions (Babaeekho et al. 2009), nevertheless the gene does not bear more polymorphism in comparison with merozoite surface protein (MSP) and circumsporozoite protein (CSP) genes. Therefore PvDBP is supposed to be a trustable candidate for *P. vivax* vaccine (Premaratne et al. 2011; Valizadeh et al. 2016). Learning about polymorphism of PvDBP gene guides us to understand which allele is more important as a specific target to produce a vaccine. Some studies showed that domain II of DBP is rich of cysteine and bears the most variable region of gene (Sousa et al. 2010). In this study

we tried to identify the prevalent alleles of PvDBP amongst 30 Iranian *P. vivax* infected cases using PstI and RsaI restriction enzymes. These enzymes were used due to producing appropriate size of the produced fragments by enzymes in order to identify better on the agarose gel as well as their availability in the markets. Results showed that 93.3% of the samples were cut at the same fragment, indicating limited PvDBP variations among the studied samples. Domain II of the gene plays an important role in connecting to host red blood cells. In this study the mentioned domain contained 170 amino acids with 7 cysteine residuals similar to those reported by Gosi et al. (2008). Four isolates as bah1, bah2, bah3 and bah4 were identified in this study and submitted to GenBank. Amount of homology among the above Iranian isolates in comparison with sal1, Indian and Korean isolates (SK₁ and SK₂) showed that the maximum and minimum identity between them were 99.82 and 82.42% respectively. While, the amount of identity amongst the isolates of this study those were registered in GenBank was more than 96.2%, indicating less variation within the Iranian PvDBP isolates. Nucleotide sequence alignments revealed that there were some substitutions in the samples in comparison with sal1, it is important to note that non-synonymous single nucleotide polymorphisms (nsSNPs) can code amino acid changes in their corresponding proteins. These nsSNPs can influence protein function, a fact that could lead to changes in antibody responses. Investigation about the effects of amino acid substitutions on protein structure and function is important for revealing the complex mechanisms of parasite caused by single amino acid mutations. Results of this study indicated that some of the obtained polymorphism from Iranian isolates led to changing in amino acids of protein chains in comparison with sal1. Somewhere a basic-polar amino acid like arginine and lysine substitute instead of a neutral-polar like serine or acidic-polar like glutamic acid. In another place a neutral-polar amino acid (asparagine, tryptophan and isoleucine) has substituted for a basic-polar amino acid (lysine, arginine and lysine). It is important to note that such replacements can be resulted in some changes in protein characterization. If a particular position was crucial for protein folding, it is important to understand that such changes may result in more alteration in its shape. Moreover these substitutions can also affect protein–protein interactions in the host body. For example, the amino acid substitutions located at the binding border or dynamic site cleft could block passing across the active site, affect the identity, change the specificity, or shift the binding affinity. Such process guides us that the mentioned data is being useful for relevant vaccine studies.

Conclusion

This study showed that there was high level of identity between submitted isolates in this study with Sall isolate. However in nucleotide level there were few single polymorphisms among isolates in comparison with Sall resulted to new substitution of amino acids. Results of this study offer that the structure-based analysis can suggest valuable information for considering the molecular mechanisms of parasite behavior to investigate about producing a vaccine with a wide range of effects.

Acknowledgements The authors would like to thank Mr. Hamid Azariyan and Leila Farivar for their valuable field and laboratory cooperation. Financially this study was supported by School of Public Health, Tehran university of Medical Sciences (TUMS).

Authors' contribution MN, AMH, BN and ZS were responsible for designing the study; collection of data; coordination and implementation of the study; BN and AMH were responsible for laboratory testing and interpretation of results; MN and ZS were primarily responsible for analyzing and interpreting the results and drafted the manuscript; MN, AMH, BN critically revised the manuscript for content. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval The study was approved by the ethical committee of research affairs of School of Public Health, Tehran University of Medical Sciences under the Registration Number of 240/1220.

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