Polymorphism analysis of malaria susceptibility biomarkers in G6PD deficiency patients

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Abstract

Background: Several studies suggested that some traits and polymorphisms in human genome such as G6PD deficiency and other genes have protective effects on susceptibility to malaria infection.

Methods: In present study we investigated the prevalence of TNFα-244G→A, TNFα-308G→A,TNFα-238G→A, NOS2-954G→C, MBL54G→A, MBL 57G→A, MBL IVS-I-5G→A polymorphisms and G6PD variants (Mediterranean, Chatham, Cosenza, A-(202,376) in 315 subjects with G6PD deficiency and 10 malaria patient. All the 315 subjects were selected from five provinces (Fars, Khuzestan, Esfahan, Yazd and Kerman) and screened by PCR-RFLP method.

Results: The NOS2-954G→A consisted GG(40.31%), GC(53.01%), and CC(6.66%) where as TNFα-308 consisted GG(68.8%), AG(31.11%) contents. The TNFα -244 showed GG(94.60%), AG(5.39%) genotypes and the TNFα-238 had GG(92.69%), AG(6.66%), AA(0.63%) genotypes. The MBL54 polymorphism had GG(75.55%), AG (24.44%), AA(0.63%) genotypes. In MBL 57, had GG(95.23%), AG(4.76%), AA (0.63%) genotypes. The G6PD variants was indicated that Mediterranean mutation in Fars, Khuzestan, Esfahan, Yazd and Kerman provinces was 79.4%, 58%, 83/8%, 64% and 63% respectively and also, the Chatham mutation was 8.8%, 8% 4.5%,3.6% and 0% respectively. Analysis of other four mutations (Cosenza, Arures and A-202 and A-367) showed that none of them had those mutations.

Conclusion: Our results suggested that genotypes which causes protection against malaria or reduction of risk for celebral malaria and death has the maximum prevalence in samples taken from the five provinces, but in the kolmogorov-smiranov test results, only NOS2-954G→C supported the theory of relation between these polymorphisms and protection against malaria.

Keywords: G6PD, polymorphism, PCR-RFLP, TNFα, MBL2, NOS2, mediterranean, Chatham and Cosenza.

Introduction

Malaria is an infectious disease caused by protozoan parasites. More than half of the world's population in approximately 100 countries is exposed to malaria with about one million deaths annually [1-3]. Iran is located in the Eastern Mediterranean region, where about 45% of the population live with the risk of having both falciparum and vivax malaria. The malaria endemic areas of Iran are located in the south-eastern part of the country, bordered in

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the south by the Persian Gulf and the Gulf of Oman and to the east by Afghanistan and Pakistan. The south-eastern part of Iran consists of the Sistan and Bluchistan province, the Hormozgan province and the tropical part of Kerman province with a combined population of approximately three millions and is considered to be a "refractory malaria region". Annual Parasite Incidence (API) was reported to be 8.74 per 1,000 population [1]. High frequency of some traits in malaria endemic areas suggested the strong selective pressure of malaria on human genome. These traits consist of: thalassemia, HLA antigens, and G6PD deficiency which are common in many areas, and have been shown to be related with the reduced susceptibility to severe malaria and the G6PD deficiency is indicated to be more important than others. This gives the selective advantage against sever malaria since prevalence of the G6PD deficiency has had 5%to 25% increase in comparison with other areas in malaria endemic area. The glucose 6-phoshphate dehydrogenase is X-chromosome linked expressing in all tissues. This is the first enzyme of pentose phosphate pathway with 5-carbon sugar ribose and NADPH synthesed by coupled oxidation /reduction reactions. Moreover this enzyme is highly polymorphic in humans by which more than 160 different mutations have been identified so far. In addition, genetic variations in several other genes, including TNFα, MBL and NOS2 have been identified. The tumor necrosis factor-alpha (TNFα) is a key cytokines with an important role in pathogenesis of severe infectious diseases. The genetic susceptibility to severe form of malaria (cerebral malaria) is associated with TNFα promoter polymorphisms and high circulating levels of cytokines. Nonetheless the level of nitric-oxide (NO), which is related to polymorphisms in NOS2 gene, could play an important role in severity of many diseases including malaria. Mannose-binding lectin (MBL) is thought to play an important role in the innate immune defense. The

ligands of MBL are high in mannose and N-acetylglucosamine oligosaccharides that presented on a variety of microorganisms. At present study, we investigate the frequency of common polymorphisms in NOS2 (1polymorphism), MBL2 (3polymorphisms), TNFα (3 polymorphisms) genes and G6PD(4 variants) which is related to protection against malaria in 315 healthy samples with G6PD deficiency and 10 samples of malaria patients without G6PD deficiency in five provinces (Fars, Khozestan, Esfahan,Yazd and Kerman) [13,31].

Methods

Study population and area: We collected 315 samples from non related healthy subjects with G6PD deficiency and 10 samples from patients who were hospitalized with the sign of malaria and their blood smears showed prescence of the parasite. All samples were collected from Fars (34 samples), Khuzestan (100 samples), Esfahan (62 samples), Yazd (55 samples) and Kerman (64 samples) provinces of IRAN which comprised 30% of disease incidence of country. Informed consent was obtained from the subjects before sampling. All of the 315 samples had G6PD deficiency using dye reduction test.

DNA extraction: Genomic DNA was extracted from white blood cells using standard methods of DNA extraction (Phenol Cholorophorm and southing out).

DNA polymorphism analysis: All collected samples including the Mediterranean, Chatham, Cosenza, Aures and A-(376,202) variants were evaluated by DNA amplification with specific primer and digestion with restriction enzyme endonuclease (Table1). Amplification of DNA samples from Mediterranean, Chatham,Cosenza, A-(376,202) and Aures variants were 583bp, 208bp, 548bp,295bp,108bp and352bp, respectively.To detect the genetic polymorphism of the NOS2-954 a 680-bp fragment of NOS2 promoter region was amplified. To detect TNFα

Table1. The polymorphisms and variants with specific primers and restriction enzymes.

Polymorphism and Variants	Primer sequence	Annealing temperature (°C)	Restriction Enzyme
G6PD-med	Med- F 5'CCCCGAAGAGGAATTCAAGGGGGT-3' Med- R 5'-GAAGAGTAGCCCTCGAGGGTGACT-3'	61	MboII
G6PD-Chat G1003A	Chat-F 5' CAA GGA GCC CAT TCT CTC CCT T 3' ChatR5'-TTCTCCACATAGAGGACGACGGCTGCCA AAGT-3'	60	BstXI
G6PD-Coz G1367C	Coz F-5'-GCA GGC AGT GGC ATC AGC AAG -3'	64	Bsu36I
G6PD A⁻ (G202A)	Coz R: 5'-GGG AAG GAG GGT GGC CGT GG -3' A202 F 5'-GTGGCTGTTCCGGGATGGCCTTCTG- 3'	56	Nla III
G6PD A⁻ (A375G)	A202 R :5' CTTGAAGAAGGGCTCACTCTGTTTG-3' A375 :5'CTGTCTGTGTGTCTGTCTGTCC3' A375R: 5' GGCCAGCCTGGCAGGCGGGAAGG3'	61	FokI
G6PD Aures	CAGCCACTTCTAACCACACACCT 3 5'-CCGAAGCTGGCCATGCTGGG 3	58	Bgl II
TNFα-308G→A	TNFα1:5'-GGCAATAGGTTTTGAGGGCCATG-3` TNFα 2:5'-CACACTCCCCATCCTCCCTGATC-3`	58	NcoI
TNFα-244G→A	TNFα 1 and TNFα 2	58	HpyF31I
TNFα-238G→A	TNFα 1 and TNFα 2	58	BspPI
MBL 54G→A	MBL1:5'-CAGGCAGTTCCTCTGGAAGG-3` MBL2:5'-GCACCCAGATTGTAGGACAGAG-3`	60	BshNI
MBL 57G→A	MBL1 and MBL2	60	MboII
MBL IVS-I-5G→A	MBL1 and MBL2	60	Hin1II
NOS2-954G→C	NOS2 1:5`-TGTTGGGACGGTGAGATCAAGGT-3` NOS2 2:5`-CTCATCAAAGGTGGCCGAGAGAT-3`	62	Eco31I

polymorphisms a 117-bp fragment of promoter region was amplified and next to detect MBL2 polymorphisms a 340-bp fragment amplified. PCR amplification was done using 2μl of DNA in a total volume of 50μl reaction mixture containing appropriate primers (Table1). The amplified products were analyzed on 10% agarose gel and visualized with ethidium bromide staining. Eight micro liters of each amplified products was digested with 15 μl of reaction mixture containing 1U of appropriate restriction enzyme (Table1). Inactivation of enzyme was performed with proteinase K after 1-12 hours incubation and the digestion solutions analyzed on 12% acrylamide gel and later visualized with silver-nitrate staining.

Statistical analysis: the two-sample kalmogorov-smiranov test was used to compare the distribution of host genetic variants in individuals with and without malaria.

Results

All the 315 subjects were screened through PCR-RFLP analysis for TNFα-244G→A, TNFα-308G→A, TNFα-238G→A, NOS2-954G→C, MBL54G→A, MBL 57G→A, MBL IVS-I-5G, G6PD-med, G6PD-Chat, G6PD-Coz, G6PD A-(G202A), G6PD A-(A375G) and G6PD Aures. The frequency of NOS2-954G→A showed that 127 out of 315 (40.31%) healthy samples had GG genotype, 167 samples (53.01%) GC and 21 samples (6.66%) CC. All the 10 samples from malaria patients showed GG genotype. In TNFα-308, 217 out of 315 (68.8%) samples showed GG genotype, 98 samples (31.11%) AG genotype but none had AA. For TNFα-244, 298 of samples (94.60%) showed GG genotype, 17 samples (5.39%) AG genotype and
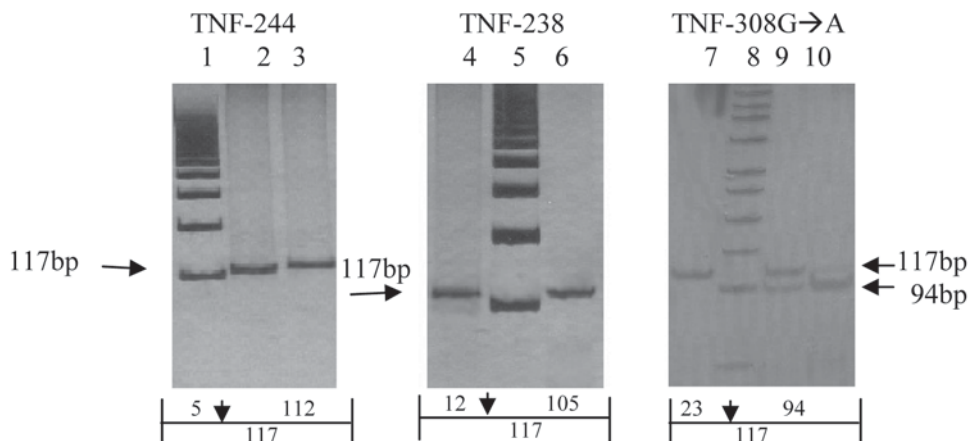
Fig. 1. Restriction digestion analysis of PCR products for TNF-244G→A, TNF-238G→A and TNF-308G→A polymorphisms with HpyF31I, BspPI and NcoI enzymes respectively on acrylamid gel. There are 117 pb fragment in all three polymorphism. Lane 2,3:GG genotype of TNF-244 polymorphism with 117 bp ; Lane 4,6: GG genotype of TNF-238 polymorphism with 117bp; Lane9,10: AG and GG genotypes of TNF-308 polymorphism with 117bp,94bp,23bp and 94bp,23bp respectively; lane 1,5 and 8: Size marker of DNA (100bp ladder).

none had AA. All malaria patients showed GG genotype in this region. In TNFα-238, 292 of samples (92.69%) showed GG genotype, 21 samples (6.66%) AG genotype and 2 of the samples(0.63%) AA genotype and all of malaria patients had GG genotype in this region. In MBL54, 238 samples (75.55%) had GG genotype, 77 samples (24.44%) AG genotype and 2 samples (0.6%) AA genotype. 2 samples of

malaria patients (20%) had AG genotype and 80% GG genotype. In MBL 57, 300 samples (95.23%) had GG genotype, 15 samples (4.76%) AG genotype and 2 samples (0.63%) AA genotype. eight samples (80%) of malaria patient GG genotype and 20% AG genotype. in MBL IVS-I-5G→A, All 315 samples showed (100 %) GG genotype and all malaria patients showed GG polymorphism. In studding of
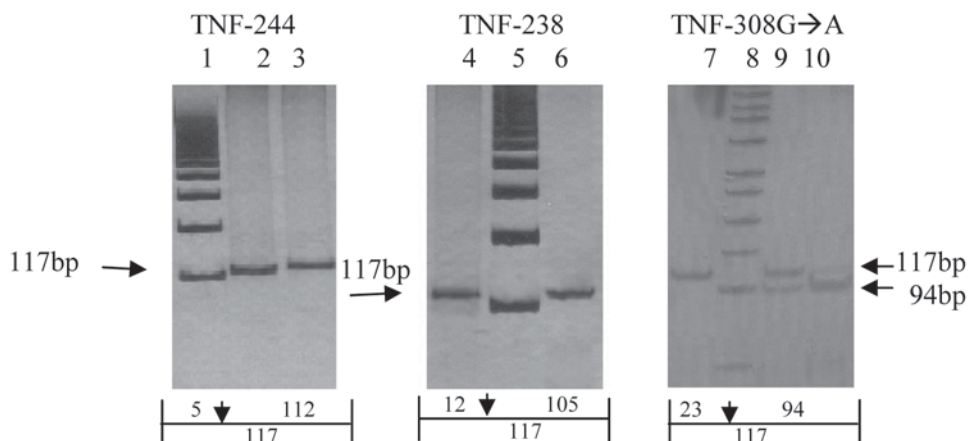

Fig. 2. Restriction digestion analysis of PCR products for MBL IVS-I-5, MBL54 and MBL57 polymorphisms with Hin1II, BshNI and MboII enzymes respectively on acrylamid gel. All of them have 340 pb fragment as a PCR product. Lane 1,2: AG genotypeand PCR product of MBL IVS-I-5 polymorphism with 240, 71, 28bp and 340bp respectively; Lane 5,6,7: GG,AA and AG genotypes of MBL54 polymorphism with (340bp), (256,84) and (340,256,84) 287,62; Lane 8,6: GA and GG genotype of MBL57 polymorphism with (340,287,62) and 340bp; lane 3,4 and10: Size marker of DNA (100bp ladder)
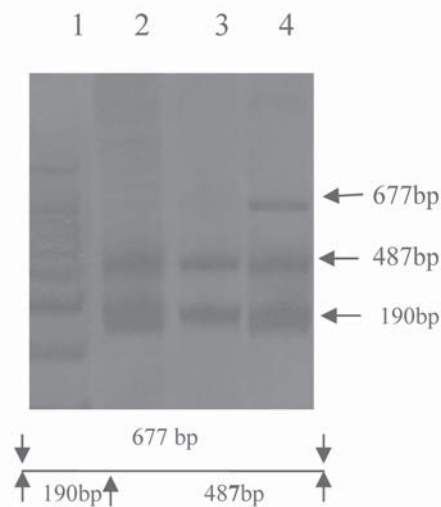
Fig. 3. Restriction digestion analysis of PCR products for NOS2-954G→C polymorphism with Eco31I enzyme on acrylamid gel. Lane 2,3: GG genotype of NOS2-954G→C polymorphism with 487and 190bp, Lane 4: CG genotypes of NOS2-954G→C polymorphism with 677,487, 190bp and Lane 1: Size marker of DNA (100bp ladder).

G6PD variant indicated that Mediterranean mutation in Fars, Khuzestan, Esfahan, Yazd and Kerman provinces was 79.4%, 58%, 83/8%, 64% and 63% respectively. Also, Chatham mutation was 8.8%,8% 4.5%, 3.6% and 0% respectively. Finally, other four mutations analyzed were Cosenza, A-202(G→A), A-
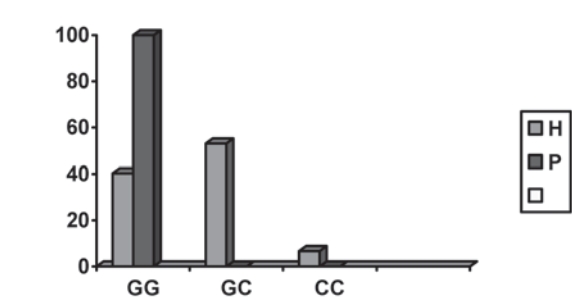

Chart 1. Distribution of NOS2-954 alleles in healthy individuals and patients.

367(A→G) and Aures (T→C) in which none had these mutations.

Difference between distribution of each genotype variants in healthy and patients objects was studied by two-samplekalmogorov-smiranov test, to find the relation between these genotype and protection against malaria. Only distribution of NOS2-954C (p=0.002 by two-samplekalmogorov-smiranov test) supported this theory.

Discussion

Malaria is known to be a dangerous and tremendously successful pathogen that is responsible for 300 million cases of infection with and one million deaths annually [3,4]. Plasmodium falciparum is the causative agent
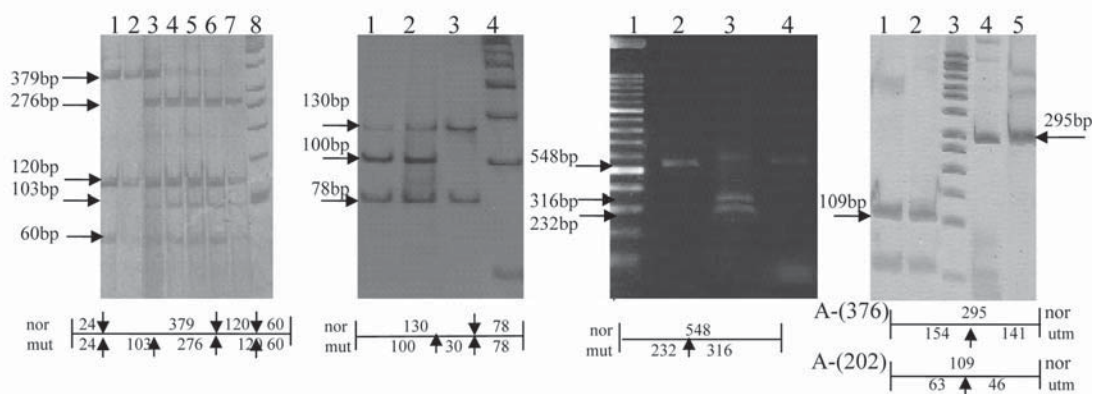

Fig. 4. Restriction digestion analysis of PCR products for G6PD Mediterranean, Chatham, Cosenza, A-376, A-202 mutation with length of 583bp, 208bp, 548bp, 295bp, 108bp and 352bp, respectively, as a PCR products and was digested by MboII, BstXI, Bsu36I, FokI and Nla III enzymes on acrylamid and agarose gel.
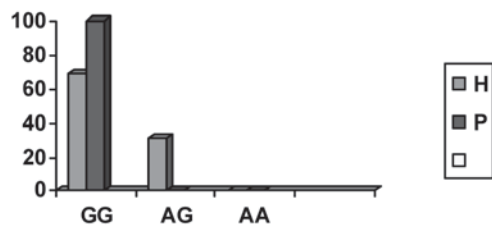
Chart 2. Distribution of TNFα-244 alleles in healthy individuals and patients.

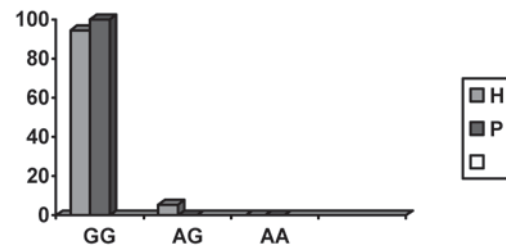
Chart 3: Distribution of TNFα-308 alleles in healthy individuals and patients objects.

in more than one million children in Africa. Other species of this parasite, p. vivax, p. Malariae, p. Ovale, can also infect humans. The genetic diversity of the parasite, generated by its sexual stage, provides the organism with many opportunities to maximally adapt to host defenses and continue transmission. Due to the length of exposure time, and significant effects on morbidity and mortality by infection, it has exerted a strong selective pressure on the human genome, and causes mutations in human genes which promote survival in areas where malaria is endemic [33]. Protection against this microorganism is provided with the polymorphisms generated in the human genome after many generations [32]. Large numbers of these polymorphisms have been found in genes which codes erythrocytes protein and cytokines. The relation between some human traits and severity of malaria infection, has been well documented [34]. These traits mainly found in red blood cell disorders like sickle cell anemia, thalassemia and other traits like G6PD

deficiency [35,36]. The protection against malaria is suggested to be related to polymorphisms in more than 30 genes [1]. Investigations have indicated that a number of variants are involved including those of the major histocompatibility complex and a cytokine-gene cluster on Chromosome 5q31-q33 [38, 39].

The TNFα codes by TNF genes located in MHC class III nearby MHC class I and II, and is a pro-inflammatory cytokine that plays an essential role in the protection against many infections including malaria, but also fatal when produced in excess [41, 42]. A study on Burkina Faso families revealed the linkage of mild malaria to the MHC region genes with a peak close to TNF [43]. Varity of polymorphisms in promoter region of TNF gene are related to severity of infectio. It has revealed that Gambian childrens who harbor homozygous TNF-308A allele are more susceptible to cerebral malaria [9]. Other studies in Gabon, Serilanka and kenia suggested individual who carries this allele are more susceptible to recurrence of
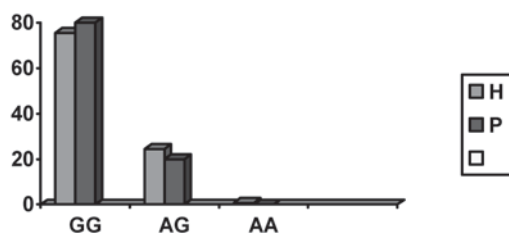

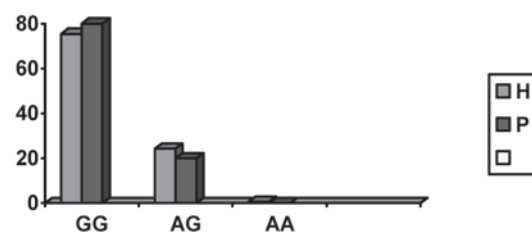Chart 4. Distribution of MBL254 alleles in healthy individuals and patients.

Chart 5: Distribution of TNFα-238 alleles in healthy individuals and patients.
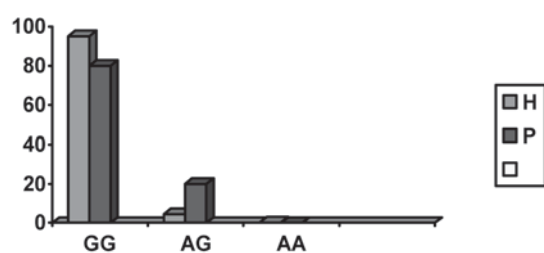
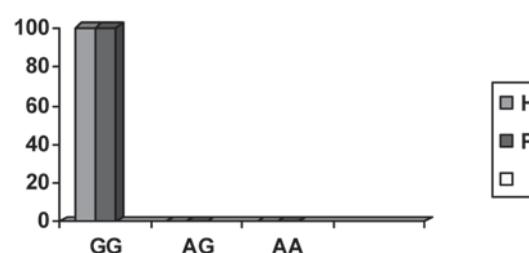Chart 6. Distribution of MBL57 alleles in healthy individuals and patients.

Chart 7. Distribution of MBL IVS-I-5 alleles in healthy individuals and patients.

plasmodium infection or death [45,46]. The TNF-376A play role in binding to transcription factor, OCT-1 and susceptibility to the cerebral malaria, and the TNF-238A is related to susceptibility to the malarial anemia. Nevertheless, results about the role of TNF polymorphisms in protection against malaria are conflicting [9, 47].

Several findings suggested single nucleotide polymorphisms (SNPs) in 5` regulatory site of TNF gene and in coding region of FCGR2A are associated with susceptibility to plasmodium falciparum Malaria [9,49,50,52]. The tumor necrosis factor alpha (TNF) have role in many inflammatory responses and also play an important role in pathogenesis of many infectious diseases such as plasmodium falciparum Malaria [10]. Transcription of TNF gene is complex and well regulated [55], and it has revealed that SNPs in 5` regulatory region of this gene are related with variety of infectious and inflammatory diseases [56,57]. SNPs in -1031, -857, -376 (1800750,G>A), -308 and -238 are located in regulatory region of TNF gene and associated with TNF production as well as protection against Malaria in different populations [50,52,49,60.61]. Taking this, it seems important to discover TNF special responses. A study on polymorphisms of TNF-enhancer and gene for FcγRIIa correlate with the severity and diversity of falciparum malaria in the ethnically diverse Indian population [61]. This differences has been also reported in 17 regions of Africa and Burkina Faso [62,21,25].

In our study three polymorphisms in promoter region of TNFα gene were analyzed based on the two-samplekalmogorov-smiranov test.

Comparison of genotype frequency for the TNFα polymorphisms, between healthy people and malaria patient revealed no significant difference ($p > 0.01$).

Several studies suggested that the presence of the different variant alleles in the MBL gene is associated with an increased tendency for infections [7,27,28]. According to two-samplekalmogorov-smiranov test, no association in frequency distribution of MBL variant alleles and susceptibility to malaria infection was found. A study among Gambian children also, has shown no relation between MBL deficiency and malaria [29]. Another study among young Gabonese children has found a weak correlation between the MBL deficiency and severe malaria [23]. This study also suggested the MBL deficiency may not be associated with malaria, but it could be a risk factor for severe malaria in children who lack well developed protective acquired immune responses [30].

Nitric oxide has been determined to possess antiparasitic activity [31], and our results shown an association between NOS2-954GC alleles and susceptibility to malaria ($p=0.002$ by two-samplekalmogorov-smiranov test). A study among Gabonian individuals shown heterozygous carriers for NOS-G954C are protected against malaria as effectively as sickle cell trait [8]. Another study showed no significant difference in multiplicity of infection between

children who were heterozygous for NOS2-954C and those with wild type alleles [23].

We found that the G6PD Mediterranean, was the most common G6PD deficient variant in these five provinces, which accounts for 69.56% of the 315 analyzed samples. Similar to other parts of Iran[13,23], in the majority of the people in these provinces ,favism is most probably due to Gd-Med, which may suggest a common origin for the populations in Iran and the Mediterranean. It should be emphasized also that G6PD Mediterranean frequency reached 91.2% in Kermanshah province [23]. Our data showed that on the average, in Iranian population, the Chatham mutation was the next one in five provinces, and accounts for 4.95%[23].Although the origin of the Iranian population is rather uncertain, but the closer similarity of the mutational spectrum to Italian (80-84% for Mediterranean, 20% for Chatham and 1.9% for Cosenza) rather than Middle East population may indicate a common ancestra origin [7-25,3-34].

Acknowledgments

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