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High-resolution Melting to Identify Single Nucleotide Polymorphisms of IL-12 Receptor Gene (IL-12RB1) in the Tunisian Population

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ABSTRACT Currently, genetic variations, especially single nucleotide polymorphisms (SNPs), have been increasingly investigated to find a potential association between these mutations and diseases in populations with different ethnicity. Therefore, rapid and efficient genotyping technologies are needed to detect and confirm these variations. This work aims to apply a High-resolution melting method to identify two IL-12 Receptor B1 gene polymorphisms in healthy Tunisian population and compare their genotypic distribution with other populations. DNA was extracted from 141 healthy volunteers enrolled in this study and genotyped for rs401502 and rs11575934 using the HRM method. We were able to detect correctly all the genotypes of the SNPs of interest with similar accuracy than DNA sequencing, using the HRM method. Minor allele frequencies of rs401502 and rs11575934 polymorphisms in the Tunisian general population are 23.8 percent and 29.8 percent, respectively. Allelic and genotypic distributions of these SNPs were found to be different from other ethnic groups. This work has enabled the establishment of a rapid, sensitive and inexpensive genotyping technique qPCR-HRM to detect genetic variations in a large series of samples. A comparison of the genotyping results of these two polymorphisms in our cohort with other populations reflects the ethnic-specific distribution.

INTRODUCTION

Interleukin 12 (IL-12) is a pro-inflammatory cytokine secreted by activated phagocyte and dendritic cells. It plays an important role in promoting Th1-type immune responses and cell-mediated immunity. Indeed, it stimulates the proliferation of activated T and NK cells, induces the production of cytokine, particularly IFN-γ and enhances the cytotoxic activity (Trinchieri 1998). IL-12 acts by binding to its receptor (IL-12R) with high-affinity. IL-12R is composed of two distinct subunits: IL-12Rβ1, encoded by IL-12RB1 and IL-12Rβ2, encoded by IL-12RB2 (Robinson 2015). IL-12Rβ1 is a type 1 transmembrane receptor that contributes to both IL-12/IL-23 signaling pathways (PRESKY 1996). The IL-12RB1 gene seems interesting as a candidate gene for testing the susceptibility to some diseases including cancer and infectious diseases. In fact, several studies have been conducted to explore the association between IL-12RB1 polymorphisms and some diseases such as cervical cancer (Hussain et al. 2013); breast cancer (Quan et al. 2014); tuberculosis (Altare 2001; Remus 2004; de Beaucoudrey et al. 2010; Boisson-Dupuis et al. 2011; Alinejad Dizaj et al. 2018; Zhou et al. 2019); Malaria (Sortica 2012; Zhang et al. 2010).

Among the reported studies on IL-12RB1 mutations, none has previously investigated the genetic distribution of rs401502 and rs11575934 among the Tunisian population. Indeed, studies on SNPs should be replicated in different populations because a genetic association of polymorphisms within a specific ethnic group through

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case-control study cannot be extrapolated to a second one, due to ethnic-specific distribution.

Several techniques have been applied for SNP genotyping namely Polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) (Lee et al. 2005; Tang et al. 2008; Wei et al. 2015), Sanger sequencing (Iskakova 2014), or Real-Time PCR-based TaqMan chemistry (Rao et al. 2014). However, these techniques are often laborious, expensive and/or time-consuming. A relatively new approach was developed, quantitative PCR followed by high-resolution melting (qPCR-HRM), which combines real-time amplification and melt curve analysis (Rouleau et al. 2009). This method is based on the analysis of the melting profile of PCR products, using saturating intercalating dyes to monitor the transition from double-stranded DNA (ds) to single-stranded DNA (ss) through gradual temperature increase (Vossen 2017).

The main advantage of HRM analysis is that it is a closed-tube method, so it does not require post-PCR sample handling. Thereby, it prevents potential PCR-product contamination (Wittwer 2003; Zhou et al. 2004; Herrmann et al. 2006; De Leeneer et al. 2008; Pereyra et al. 2012). The melting profile of a PCR product depends on its GC content, length, sequence and heterozygosity (Tamburro and Ripabelli 2017; van der Stoep et al. 2009; Liew et al. 2004; Reed 2007).

Objectives

In the present study, we only included healthy Tunisians to develop a real-time PCR followed by HRM analysis for genotyping of two SNPs within the IL-12RB1 gene (rs401502 and rs11575934). Because of intra and inter-ethnic in differences allelic-difference distribution, we aim to establish the allelic frequencies and genotype distribution of each studied SNP in the Tunisian general population and compare it with other ethnic groups.

METHODOLOGY

Study Samples and the Ethical Statement

One hundred forty-one healthy subjects, collected from external consultants service; Pasteur Institute of Tunis, were included in this study**.** Ethical approval was obtained from the Biomedical Ethics Committee of Pasteur Institute of Tunis (reference number: IPT/PCI-LR11IPT06/21/2013). All recruited subjects gave their agreement to participate and sign an informed consent.

DNA Extraction

Genomic DNA was extracted from whole blood samples using the salting-out method. In this technique, both red blood cells and white blood cells were lysed by repeated washings using Tris-EDTA buffer. Thereafter, the pellet was incubated in digestion buffer (Tris-EDTA, NaCl, SDS and 20 mg/ml of proteinase K) for 2 hours at 56°C. Next, a concentrated salt solution (NaCl 6M) was used to precipitate proteins. Finally, DNA was precipitated with ethanol and conserved at 20°C.

Positive Controls Detection

Randomly selected DNA among collected samples was sequenced by Sanger method to identify genotypes of two SNPs in the IL-12RB1 gene namely +1196G/C (G378R, NCBI SNP ID: rs401502) and +705A/G (Q214R, NCBI SNP ID: rs11575934). These samples with known genotypes (homozygous and heterozygous) were included as controls in all experiments of HRM.

The target sequence of each SNP was first amplified by conventional PCR, using the same primers as the qPCR-HRM assay (Table 1). Amplification was achieved in a 25 µl final reaction

Table 1: The primers of IL-12RB1 gene polymorphisms used in the study

SNP ID	*Nucleotide change*	*Amino acid change*	*Location*	*Primer sequence (5'-3')*	*PCR Product size (bp)*
rs401502	+1196G/C	G378R	19:18069603	F-GCATTGAATGGCAGCCTGTG R-GTAATGGCCTGGAATGGCCT	107
rs11575934	+705A/G	Q214R	19:18075808	F-GGTTAAGTGACTGGTGCCAAG R-CTCAAACCACTGGCCTCAAG	322

SNP ID: single nucleotide polymorphism identity; bp: base pair; F: forward; R: reverse

volume containing 1µl DNA, 0.8µM of each primer, 2.5mM $MgCl_2$, 200µM dNTP and 1.5 unit of Taq DNA polymerase. The PCR conditions were as follow: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec and extension at 72°C for 30 sec, then a final step at 72°C for 5 min.

PCR products were purified using Exo-SAP subsequently sequenced using BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems™) on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems®).

SNPs Genotyping by qPCR-HRM

IL-12RB1 +1196G/C (rs401502) Polymorphism

In order to genotype the rs401502 polymorphism, a pair of primers was designed using The *Primer 3* tool (v.0.4.0) (http://frodo.wi.mit.edu/primer3/). Add to that, to increase the specificity and efficiency of the qPCR-HRM, primers were conceived to flank only a single variation in a small sequence of 107bp (Table1). The qPCR mixture contained 1µl of extracted DNA, 6µl of Kapa Master Mix (2×), 0.25µM of each primer and sterile water to a final reaction volume of 12µl. The cycling conditions were performed on a Roche LightCycler® 480 System (Roche Life Science) as follows: 5 min at 95°C; 60 cycles of 10 sec at 95°C, 25 sec at 63°C and 25 sec at 72°C. After cycling, a melting curve program was run starting with a 95°C incubation for 1 min, followed by continuous acquisitions every 0.2°C from 65°C to 95°C. Generated Data were analyzed using the LightCycler 480 software (Roche Life Science).

IL-12RB1 +705A/G (rs11575934) Polymorphism

The +705A/G polymorphism was genotyped using the primers described by Lee et al. (Table 1) (Lee et al. 2005). The PCR reaction was performed on the Mic Real-Time PCR System. The total reaction volume was 15µl containing 1 µl DNA, 0.5 µM of each primer, 7.5µl Precision™ HRM Master Mix. The cycling conditions were as follows: 8 min at 95°C, followed by 45 cycles of 95°C for 5 sec and 66°C for 45 sec. The melting step gradually increases from 45°C to 95°C by 0.2°C/sec (with fluorescence data acquisition every 1 s). Data were acquired using the Mic Real-Time PCR Software.

Statistical Analysis

GraphPad Prism 6 software was used for statistical analysis. Allele and genotype frequencies were tested for Hardy-Weinberg equilibrium using the chi-square (χ^2) test by comparing the observed frequencies with the expected frequencies.

RESULTS

Positive Controls

We were able to identify the three genotypes (homozygous wild type, heterozygous and recessive homozygous) for tested SNPs by Sanger Sequencing. Individuals with known genotypes were used as references in HRM assay to assign genotypes for trial samples.

HRM Analysis

IL-12RB1 +1196G/C (rs401502) Polymorphism

Primer Specificity

The designed primers allowed a specific amplification of the target sequence. As shown in figure 1B a single peak was obtained at approximately 91°C. The PCR product was migrated on an agarose gel to check for the size of the target sequence.

HRM Results

The generated data were analyzed by LightCycler® 480 Software (Roche Life Science). The amplification curves are shown in Figure 1A (with Ct starting from the 26th cycle). Figure 1B shows the melting peaks corresponding to the three studied genotypes based on Tm differences (Tm between 90°C and 92°C). Samples with later amplification (Ct> 28) and /or generating unexpected melting peak (Tm) were excluded from the analysis. Thereafter, the generated melting curves were normalized by selecting a linear region before and after the melting zone. Next, an adjustment of the active melting zone was done to eliminate the fluorescence variance between samples and thus

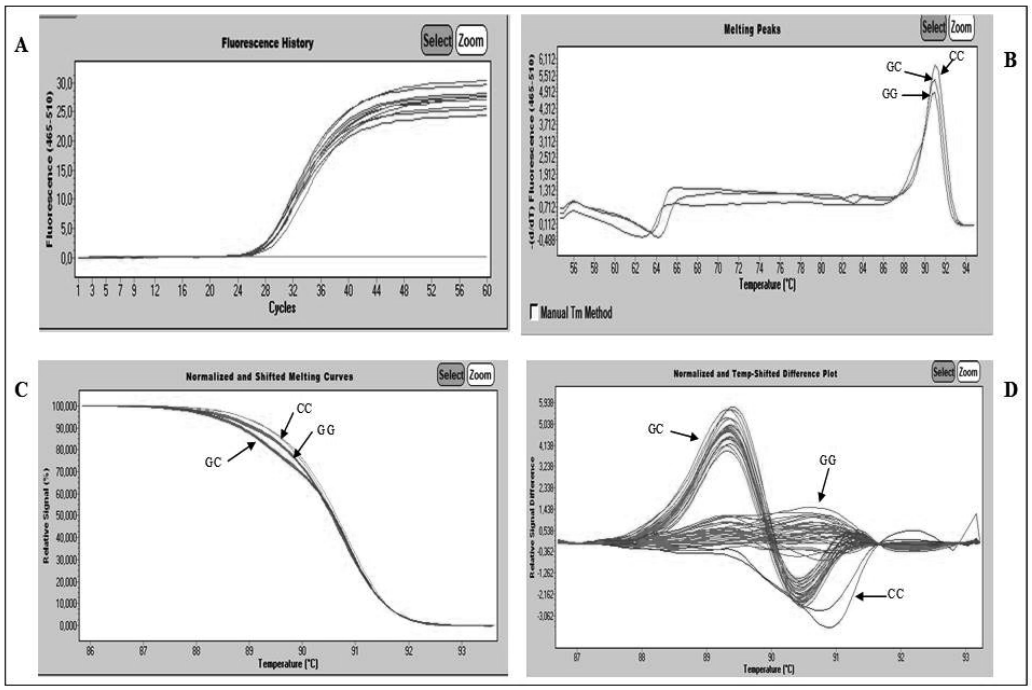

Fig. 1. HRM Analysis of IL-12RB1+1196G/C (rs401502) polymorphism. 1A. Amplification curves; 1B. Melt plot showing the Tm variance between the three genotypes; 1C. Normalized melt curves; 1D. Differential curves.

focus the analysis on the thermal denaturation behavior of DNA ds (Fig. 1C). The difference curves allowed the grouping of individuals with the same Tm and curve shape in a cluster (Fig. 1D). The genotypes were correctly assigned by referring to control samples (with known genotypes GG, GC and CC).

Then, the allele frequencies and genotype distribution analyses were performed using the chi-square (χ^2) test, as shown in Table 2.

IL-12RB1 +705A/G (rs11575934) Polymorphism

The obtained data were analyzed by Mic Real-Time PCR Software. The results are summarized in Figure 2. The target sequence containing the IL-12RB1 +705A/G polymorphism was successfully amplified with amplification curves starting from the 29th cycle (Fig. 2A). The heteroduplex (AG) was easily identified based on the shape of the melting curve. As it is a class 1 SNP (705A> G) the distinction between the wild homozygous (AA) and the recessive homozygous (GG) is easy and it is based on a shift in Tm (Fig. 2B, 2C). In Figure 2B, melting peaks profile revealed peaks (between 86°C and 87°C) corresponding to the three tested genotypes as well as small peaks at 75°C resulting probably from primers hybridization. The differential curves obtained after subtraction of the reference allowed grouping of individuals with the same genotype (Fig. 2D). The allele and genotype frequencies are shown in Table 2.

Allelic Frequencies and Genotype Distribution in the Tunisian Population

IL-12RB1 +1196G/C Polymorphism

Hardy-Weinberg equilibrium test shows that our population is in equilibrium for the IL-12RB1 +1196G/C polymorphism with χ^2=3.39 and p=0.06 (Table 2). The allelic distribution of this polymorphism is as follows: the major allele +1196G

Table 2: Allelic and genotypic frequencies of the IL-12RB1 rs401502 and rs11575934 SNPs in the Tunisian population

SNP	*Genotype frequency N (%)*			*Allele frequency N (%)*		*HWE*	
+1196G/C (rs401502)	*GG*	*GC*	*CC*	*G*	*C*	χ^2	*p-value*
	78 (55.3%)	59 (41.9%)	4 (2.8%)	215 (76.2 %)	67 (23.8%)	3.39	0.06
+705A/G	AA	AG	GG	A	G		
(rs11575934)	69 (49%)	60 (42.5%)	12 (8.5%)	198 (70.2 %)	84 (29.8%)	0.042	0.83

Abbreviation: N: Number of samples; HWE: Hardy–Weinberg equilibrium. $\div^2$: test chi-deux

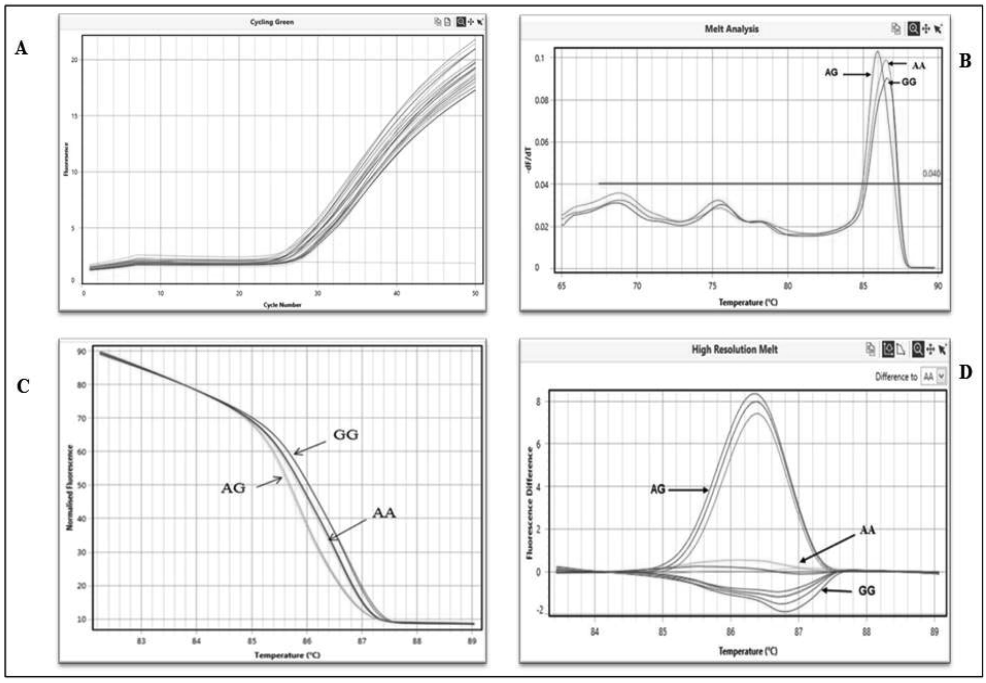

Fig. 2. HRM Analysis of IL-12RB1=705A/G (rs1575934) polymorphism, 2A. Amplication curves; 2B, Melt plot; 2C. Normalized melt curves; 2D. Differential curves

and the minor allele +1196C frequencies are respectively 76.2 percent and 23.8 percent. Therefore, the genotype distribution in the Tunisian population is as follows: GG (55.3%), GC (41.9%) and CC (2.8%). These frequencies are different from those reported in other ethnic groups. In Tunisians, the MAF is lower than the European and East Asian populations (with 31.2% and 37.6%, respectively). Whereas, it is different compared with African and American populations (17.5% and 17.3%, respectively). When we compare the genotypic distribution, we find that the heterozygous and wild homozygous frequencies in the Tunisian population are close to those of the European and East Asian populations (46.7% and 40.1% for GG and 44.1% and 44.6% for GC). However, these frequencies are greatly different from Africans and Americans: 68.4 percent (GG) and 28.3 percent (GC). Furthermore, the frequency of the recessive homozygous genotype CC is close to African and American frequencies (3.3% and 3.2%, respectively) and different from the Eu-

ropean and East Asian populations (9.2% and 15.3%).

IL-12RB1 +705A/G Polymorphism

Hardy-Weinberg equilibrium test shows that our healthy population is in equilibrium for the IL-12RB1 +705A/G polymorphism ($\chi 2 = 0.042$, $p = 0.83$) (Table 2). The allelic and genotypic frequencies are shown in Table 2. The allelic frequency in wild type (A) was found to be 70.2 percent and 29.8 percent in the mutant allele (G). The frequencies of AA, AG and GG genotypes were 49.0 percent, 42.5 percent, and 8.5 percent, respectively.

The MAF in our Tunisian cohort is close to the European ethnic group (31.1%), slightly lower than the East Asian population (with 37.3%) and significantly different from African and American populations (9.9% and 16.3%, respectively). The genotypic distribution in the Tunisian population is close to Europeans: AA (46.9%), AG (43.9%) and GG (9.2%). However, the genotype frequencies are different from other ethnic groups.

DISCUSSION

The important role of IL-12 and its receptors (IL-12R Beta 1 and IL-12R Beta 2) in the immune response justifies the interest of studying genetic polymorphisms in the genes encoding both IL-12 and its receptors. Moreover, it could explain a large number of works carried on these cytokines (van de Vosse et al. 2013; Robinson 2015). Indeed, many studies have described the association of several IL-12RB1 polymorphisms with susceptibility to certain diseases (de Beaucoudrey et al. 2010; Altare 2001; Boisson-Dupuis et al. 2011; Sortica 2012; Nekooie-Marnany et al. 2018).

In this work, the researchers developed a high-resolution melting (HRM) technique to study two SNPs within the IL-12RB1 gene, namely +1196G/C (rs401502) and +705A/G (rs11575934). They aim to establish the allele frequencies and genotype distribution of these polymorphisms in the healthy Tunisian population and establish a comparison with published frequencies data of other populations. These two SNPs have been previously genotyped using different molecular tools such as PCR-RFLP (Lee et al. 2005; Tang et al. 2008); Direct sequencing (Remus 2004) and Single-Strand conformation polymorphism (SSCP) (Lee et al. 2003). But, this is the first study that uses HRM to genotype these polymorphisms. This relatively new technique allowed SNP-genotyping in double-stranded DNA by comparing profiles of melting curves (Vossen 2017). It is a powerful molecular method that permits, thanks to its high sensitivity, a good detection of DNA sequence variations in a large series of samples without resorting to cumbersome post-PCR steps (Wittwer 2003). On top of that, HRM is a closed-tube system that does not require additional reagents or manipulator intervention after the amplification step avoiding PCR product contamination (Li et al. 2019; Perez-Baez et al. 2017; Liew et al. 2004).

Consequently, HRM analysis is considered a good means for SNP-genotyping (Al-Koofee et al. 2019). Furthermore, an effective design of the

Table 3: Comparison between allelic and genotypic frequencies (%) of IL-12RB1 gene polymorphisms in Tunisian and other populations[a]

SNP	*Allele/ genotype*	*TUN (current study)*	*EUR*	*EAS*	*AFR*	*AMR*
IL-12RB1 +1196G/C	G	76.2	68.8	62.4	82.5	82.7
(rs401502)	C	23.8	31.2	37.6	17.5	17.3
	GG	55.3	46.7	40.1	68.4	68.6
	GC	41.9	44.1	44.6	28.3	28.2
	CC	2.8	9.2	15.3	3.3	3.2
IL-12RB1 +705A/G	A	70.2	68.9	62.7	90.1	83.7
(rs11575934)	G	29.8	31.1	37.3	9.9	16.3
	AA	49.0	46.9	40.5	81.1	70.0
	AG	42.5	43.9	44.4	18.0	27.4
	GG	8.5	9.2	15.1	0.9	2.6

[a]: data from the SNP database; TUN: Tunisian; EUR: European; EAS: East Asian; AFR: African; AMR: Latin American

primers is a key step for the success of this molecular tool. In order to increase sensitivity, the amplicon should be small and shouldn't include more than one variation (only target SNP). Also, it is preferable to use the same extraction method and to work with equal amounts of target DNA in order to obtain a similar fluorescent signal and reduce the variations (Vossen et al. 2009; Rouleau et al. 2009).

Among the Tunisian population, the minor allele frequency of the IL-12RB1 gene +1196G/C polymorphism was found with 23.8 percent. Therefore, the frequencies of GG, GC and CC genotypes were 55.3 percent, 41.9 percent, and 2.8 percent, respectively. The frequencies distribution of our population was compared with other ethnic groups using frequencies data reported by the 1000 Genome Project. The minor allele frequency is slightly lower than the Europeans (31%) and East Asian population (38%). Whereas, it is different compared with African and American populations in which this allele was present with 18 percent and 17 percent, respectively.

For the IL-12RB1 +705A/G polymorphism, the minor allele IL-12RB1+705G was present in the Tunisian general population with 30 percent. This frequency is close to that found in the European population (31%) and slightly lower than the Asian population (37%). However, it is far from the frequencies obtained for the African and American populations (10% and 16% respectively). These dissimilarities could be explained by the existence of a genetic background specific to each population. This finding highlights the necessity of reproducing SNPs association studies in different ethnic groups.

Also, our results reflect the fact that the Tunisian population is genetically heterogeneous due to numerous civilizations that have settled in Tunisia throughout its history (Kefi et al. 2015).

CONCLUSION

This work has enabled the development of a genotyping technique "qPCR-HRM" allowing, thanks to its good sensitivity, the detection of genetic polymorphisms in a large series of samples. Finally, a comparison of the genotyping results of these two polymorphisms in our population with other studied ones reflects the ethnic-specific distribution.

RECOMMENDATIONS

The developed technique "qPCR-HRM" could be used to genotype other polymorphisms among healthy and patients with cancer in a large series of samples.

Frequency data of the Tunisian population could be exploited in a case-control study to evaluate the association between these genotyped SNPs and cancer.

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LIST OF ABBREVIATIONS

- AFR: African
- AMR: Latin American
- EAS: East Asian
- EUR: European
- HRM : High-Resolution Melting
- HWE: Hardy–Weinberg equilibrium
- IL-12 : Interleukin-12
- IL-12R : Interleukin-12 Receptor
- IL-12RB1 : IL-12 Receptor Beta 1 gene
- MAF: Minor Allele Frequency
- PCR: Polymerase Chain Reaction
- RFLP: Restriction Fragment Length Polymorphism
- SNP : Single Nucleotide Polymorphism
- TUN: Tunisian

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