Association of rs12913832 in the HERC2 Gene Affecting Human Iris Color Variation

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ABSTRACT

Introduction: Human eye colour as a physical trait is based on the developmental biology and genetic determinants of the structure known as the iris, which is part of the uveal tract of the eye. Prediction of human visible characteristics (EVCs) by genotyping informative SNPs in DNA as biological witness opens up a new avenue in the forensic genetic. Variation of iris color rely on the amounts of eumelanine and pheomelanin. The aim of this research was to determine and evaluate the frequency and the association of rs12913832 with prediction of human eye color in 53 volunteer of Iranian population samples.

Methods: A selection of human body blood samples were collected from donors with informed consent in Clinic Ophthalmology of Baqiyatallah hospital. DNA was extracted from the samples using RGDE procedure. PCR primers for rs12913832 were designed to give amplicon sizes up to 189 bp and Single base extensions (SBE) were done by applying the SNaPshot Multiplex kit in 6 µl reaction volumes. The results were analyzed with the SPSS 22.0 software package.

Results: The frequency of eye color were achieved for brown 34%, blue 17% and intermediate colors 49%, respectively. The genotype frequencies of T/T, C/T and C/C in our population were 4.26 %, 8.35 % and 7.37%, respectively. The statistical analysis revealed the two genotypes including T/T and C/C had a significant associate with dark brown eyes and bright blue eyes, respectively. The sensitivity and specificity of our method were determined 100% and 56.25%, respectively.

Conclusion: Our results demonstrated that rs12913832 C>T polymorphism is associated with blue iris color in Iranian population. However, assessment SNP markers by using SNaPshot is a key tool for tracing unknown persons to get primarily information about genotypic and phenotypic characteristics.

Key Words:
Forensic genetic, SNP, Perdition, Genotype, Eye color

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1. Introduction

The study of human eye color as a physical trait is based on the biology, chemistry, morphology and genetic determinants of the structure known as the iris, which is part of the tract of the eye. With information collected from this type of data, we can overcome the routine problems which occur in forensic studies [1]. In addition to hair and skin color, one of the most important EVCs studied in the FDP, is the color of the iris. Eye color often multifactorial inheritance, although in the past it was believed that this is a form of Mendelian inherited trait. Eye color is generally divided into three categories: blue, brown and intermediate. In fact, a wide range of colors in this property due to the melanin exist in the melanocytes of the iris ascending gradient from blue to brown color of eyes. So that eye color is a unique characteristic like fingerprints [2].

In order to identify genes involved in pigmentation process, meticulous studies have been done on animal models, the results indicate that about 127 genes involved effectively in the pigmentation process in mice. About 68 genes from a mice was a significant homology with human genes. The human genome contains 6 (OCA2, HERC2, TYR, etc) genes which have a special relationship in determining eye color [3]. Genome-wide association studies in people of Europeans decent have confirmed eye color as a polygenic trait, with the HERC2/OCA2 genes explaining the most of the blue and brown eye color inheritance, whereas other genes such as SLC2A4, TYR, TYRP1, SLC45A2, and IRF4 contribute additionally to eye color variation, albeit with minor effects MC1R gene and MC1R receptors are linked in the biosynthesis of melanin. In addition, MATP, OCA2 gene encode the P protein that plays an important role in the melanin biosynthesis. One of the genes involved in eye brown and blue color is HERC2 (HECT and RLD domain containing E3 ubiquitin protein ligase 2). It should be noted that this gene and markers have not been identified in the process of pigmentation, but the signs of the function of this gene in DNA repair has been demonstrated [4, 5]. It has been proved that alternative suitable marker STR in criminal trials are polymorphisms single nucleotide [SNP) [6]. SNPs in criminal studies have many advantages in comparison with STR, for example, the SNP mutation rate is less than the STR (100 times less), which makes tests for parentage and relationship very important [7].

Another point about SNPs, is that it allows the rapid, comprehensive and automatic studding. Today more than fifty SNP at the same time try to be investigated in forensic studies [8, 9]. Predicting eye color using DNA is currently available. Fan Liu and coworkers was conducted using NGS (Next Generation Sequencing) in European population via GWAS analysis showed three locus consisting of 1q42.3, 17q25.3 and 21q22.13 had associated with the eye color. Assessment study has shown that LYST genes and DSCR9 a significant role as one of the candidate areas in more than 50% of the phenotypic variance in predicting pigmentation of EVCs. Recently, Walsh and et al. in a study which was conducted in 2013 and predicted eye color evaluated and endorsed the European population. In this study, we investigated samples taken from six ethnicities in Iran for the first time we therefore expect the sensitivity of our procedures to meet the requirements of routine forensic applications in most cases. In order to use the techniques in the medical laboratory, this technique was used to assess the diagnostic sensitivity.

2. Materials & Methods

The study population comprised 53 unrelated Iranians volunteers, ethnic group including Kurdish, Persian, Azeri, Lur, Mazani and Guilaki, who signed a written consent for their DNA to be used in the study. The research was approved by the Baqiyatallah Medical Ethics Committee. The eye color was defied according to descriptions provided by the volunteers and our own grading. For confirmation and in order to prevent bias, photographs of each donor's eyes

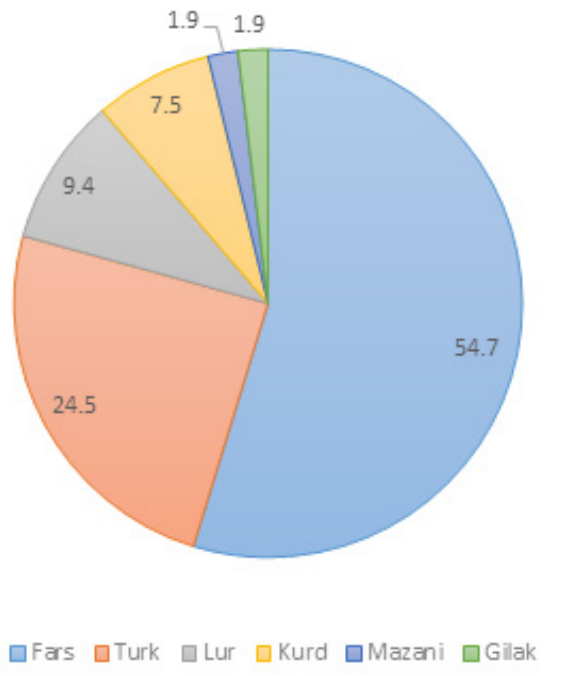

Figure 1. Ethnicity frequency (Persians, Azari, Lur, Kurdish, Mazani and Guilaki) study in percentage terms.

Table 1. Features of SNP markers used to predict eye color and design related information SBE Primer.

Allele	Concentration	SBE length	Gene	SNP
A/G	1.5	TGATGATAGCGTGCAGAACTTGACA	HERC2	rs12913832

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were taken. In the second step after taking the written consent of all volunteers, 3 ml venous blood was taken using the anticoagulant tubes k3-EDTA (Ethylene Diamine Tetra acetic Acid). Blood samples were collected from the sampling Baqiyatallah Polyclinic Hospital. Color images of the color space of the eye and particularly the iris were taken using a macro lens Using Topcon TRC-IM900 fundus camera while an ophthalmologist was monitoring. This study was approved by Ethics Committee of the Baqiyatallah University of Medical Sciences. DNA extraction was performed using standard methods RGDE [10]. To determine the concentration of extracted DNA using Nanodrop and the optical density (OD) and the ratio of light absorption (260/280) were calculated. Finally, the light absorption ratio (260/280) for all 53 samples were on average 1.6 to 1.8. For genotyping SNP marker rs12913832 (A/G) SNaPshot method was used for the first time in Iran.

Applying SNaPshot at first initial PCR with specific primers must be done to amplified area with target SNP '3 - TGATAGCGTGCAGAACTTGACA- '5 and '3 - TAATTCAAAATGCCCCCAAG'- 5. To perform initial PCR, we used 150 ng of purified DNA containing 3 µl of buffer µl30 volume X10, µl200 of non-labeled dNTP, 5.1 from Mgcl2 and AmpliTaq Gold® DNA Polymerase Enzyme, in order to assess the approved initial PCR, DNA gel electrophoresis is performed for all 53 samples in 2% agarose. The PCR program for pre-denaturation was performed at 94°C for 5 min followed by 30 cycles of 94°C for 30 seconds and 60°C for 30s, 72°C for 30 seconds and at the end of 72°C for 5 minutes. In order to ensure the accuracy of PCR reaction, 25 µl of the PCR products performing sequencing of SNP marker, have been used by Genetic Analyzer ABI 3130xl Genetic Analyzer (Applied Biosystems). In order to perform SNaPshot reaction, we designed the forward, reverse and SBE (Single Base Extension) primers (show in Table 1) and the SNaPshot Multiplex Kit (ABI Applied Biosystems P/N: 4323151) reaction mix in a total reaction volume of 5 µl was done. The protocol consists of a single multiplex two step PCR using 1 µl genomic DNA extract (varying concentrations) and primers in a 12 µl reaction which includes PCR buffer, 2.7 mM MgCl2, 200 mM of each dNTP and uses adjusted thermocycling conditions for increased specificity (1) 96°C for 5 min, 33 cycles of 95°C for 30s, 50°C for 5 s and 60°C for 30s.

Then 1 µl of PCR products using Exo/SAP-IT (USB Corporation) enzymes for purification was performed according to the manufacturer's manual. In order to prevent self-ligation in SNaPshot reaction, we treated with CIP (Calf-intestinal alkaline phosphatase) as previously published. Finally, all the products of the SNaPshot after enzymatic reaction, was electrophoresis in capillary electrophoresis device ABI 3130xl Genetic Analyzer. All cleaned products were analyzed on the ABI 3130xl Genetic Analyzer (Applied Biosystems) with POP-7 on a 36cm capillary length array. Run parameters were optimized to increase sensitivity, with an injection voltage of 2.5 kV for 10s, and run time of 500 s at 60°C.

Statistical analysis

All statistical calculations were performed using SPSS 15.0.1 for Windows (SPSS Inc., Chicago,USA). Outliers were identified as observations outside 1.5 times the interquartile range of the data.

3. Results

The study included 53 unrelated volunteers (38 males and 15 females) (Figure 1) from ethnicities (Persian, Turkish, Lur, Kurdish, Mazani and Guilaki) (Table 2).

After examination by a doctor, natural color of the iris and other environmental factors including the lack of specific drug, the presence or absence of the iris and conjunctival

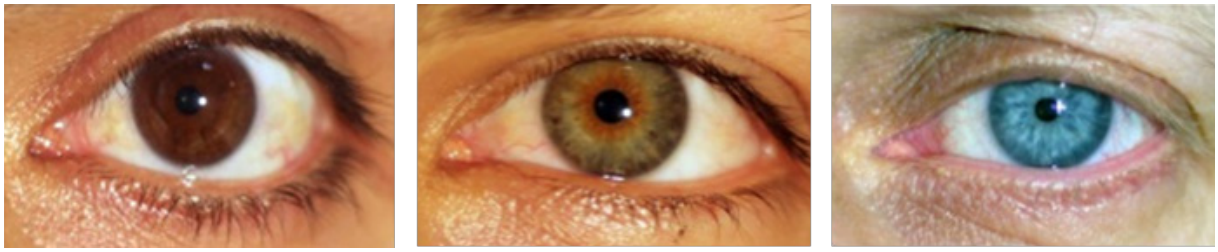

Figure 1. Eye color photos of three different volunteer.

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Table 2. Distribution of volunteers according to a two-stage diagnostic criteria established for inclusion.

NO	Sex	Ethnic	Eye color	rs12913832
1	Male	Lur	Intermediate	CT
2	Male	Turk	Dark	CC
3	Male	Turk	Dark	TT
4	Male	Lur	Dark	TT
5	Male	Fars	Dark	CT
6	Male	Turk	Dark	CT
7	Male	Fars	Intermediate	CC
8	Male	Turk	Intermediate	CC
9	Male	Turk	Dark	TT
10	Male	Lur	Intermediate	CT
11	Male	Fars	Intermediate	CT
12	Male	Fars	Intermediate	CT
13	Male	Fars	Intermediate	CT
14	Male	Mazani	Dark	CT
15	Male	Fars	Dark	TT
16	Male	Fars	Dark	TT
17	Male	Fars	Intermediate	TT
18	Male	Fars	Dark	TT
19	Male	Kurd	Intermediate	CC
20	Female	Kurd	Dark	TT
21	Male	Fars	Dark	TT
22	Male	Fars	Intermediate	CC
23	Male	Lur	Dark	TT
24	Male	Fars	Dark	CC
25	Male	Fars	Intermediate	CT
26	Female	Fars	Dark	CT
27	Female	Fars	Dark	CC
28	Male	Fars	Intermediate	CC
29	Male	Fars	Intermediate	CT
30	Male	Lur	Intermediate	TT
31	Male	Fars	Intermediate	CC
32	Male	Fars	Intermediate	CC
33	Male	Turk	Intermediate	TT
34	Male	Turk	Intermediate	CT
35	Male	Turk	Intermediate	CT
36	Male	Fars	Blue	CC
37	Female	Turk	Intermediate	CT

NO	Sex	Ethnic	Eye color	rs12913832
38	Male	Fars	Intermediate	CC
39	Male	Fars	Intermediate	CT
40	Male	Fars	Blue	CC
41	Female	Fars	Blue	CC
42	Female	Fars	Blue	CC
43	Female	Fars	Blue	CC
44	Female	Gilak	Intermediate	CT
45	Male	Kurd	Blue	CC
46	Female	Turk	Intermediate	CT
47	Male	Turk	Blue	CC
48	Female	Fars	Blue	CC
49	Female	Turk	Intermediate	CT
50	Female	Kurd	Dark	TT
51	Female	Fars	Blue	CC
52	Female	Turk	Dark	TT
53	Female	Fars	Intermediate	CT

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diseases, etc. were examined. All volunteers participating in the study were examined by ophthalmologist under the same optical condition. Photograph of the iris (Irisgraphy) was classified with Topcon IM900 (Figure 1). The ophthalmologist classified eye color without knowledge of genetic characteristics. The results showed that 34% of volunteers have brown eyes, 49% intermediate and 17% blue. Other phenotypic data has not been considered in the arrangement of the images. Most predictions are on the basis of rs12913832 in order to predict eye color.

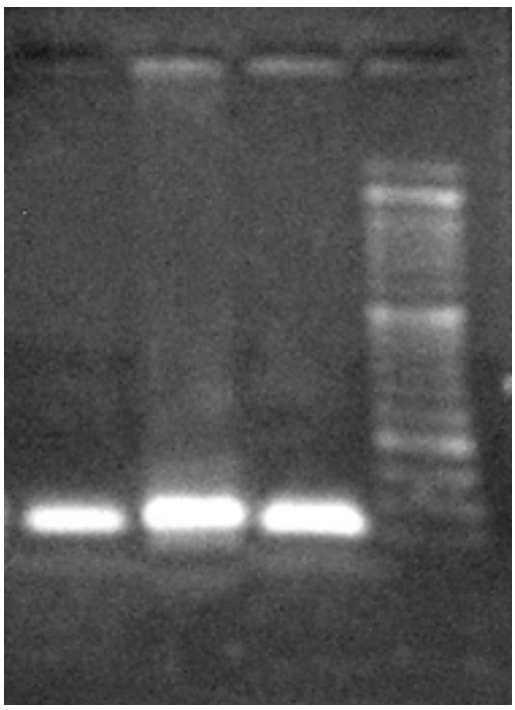

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Figure 2. Electrophoresis of 189 bp PCR product for the proliferation marker rs12913832 on 1.5% agarose gel. M: 1 Kb DNA Ladder.

In order to ensure the presence of rs12913832 allele in the genome amplification, specific primers for this area were designed. As expected, the piece 189 bp was observed on the gel after staining, the measure was consistent with our target gene. In order to ensure the sequence of amplified region, three PCR products of the volunteers were sequenced and evaluated with human genome version GRCh38: CM000675.2. The results showed that the target amplified sequence is of full compliance. The results of PCR amplification of the desired marker on 1.5 % agarose gel is shown in Figure 2.

In our study, genotype frequencies T/T that is associated with dark brown eye colors is 26.4%, and the prevalence of genotype C/T that is associated with intermediate eye color is 35.8% and genotype C/C with bright blue eye colors is 37.7%. Compare predict eye color from the rs12913832 profile and eye color revealed the sensitivity and specificity were 100% and 56.25%, respectively.

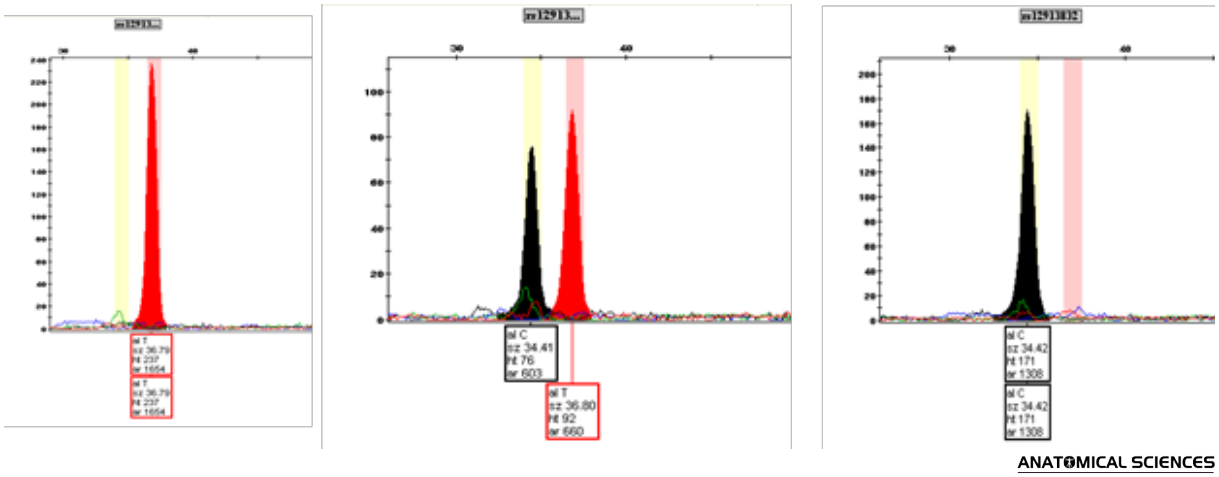

Figure 3. SNP marker rs12913832 Profile in three volunteers with different eye colors phenotype was evaluated with SNapShot [Figure 1-1: homozygous genotype TT/AA associated with dark eye color, Figure 1-2 heterozygous genotype CT/GA associated with intermediate eye color, Figure 1-3; homozygous genotype GG/CC associated with blue eyes).

4. Discussion

Notably, SNapShot fragments lengths was considered between 30 bp and less than 180 bp, allowing to us for future application in forensic samples. Polymerase chain reaction and the use of primers of different lengths (SBE) should be selected for each SNP in the genome. In the studies conducted in different populations around the world, rs12913832 marker was defined as a powerful marker to differentiate between blue eyes brown color. PCR and SBE multiplex optimizations aimed to balance all SNP alleles due to ensure genotyping accuracy in a wide range of DNA quantities [11, 12]. In this study, the association with T/T genotype with Dark eyes and T/C genotype with the intermediate colors such as brown, hazel, and green and C/C genotype was also proved with blue eyes. Therefore, the statistical results are acceptable for one marker. Considering the demography of the country, more markers are needed in order to identify the exact eye color. As in Figure 3, profiles extracted from the software Gene Mapper show that this marker can act specific in the diagnosis of brown eye color blue. The informative SNPs due to its sensitivity and the robust design, profiles could be generated from low concentrated (40 pg) and highly degraded DNA. Each forensic laboratory has the possibility to conduct this easy handling method with the standard facilities available.

The evaluation method can be used in DNA samples that are degraded such as humidity and salty environments. Multiple genes are involved in the extension of eye color variation so that further study is needed to optimize the eye color prediction particularly in the intermediate domain in which no precise prediction results can be obtained. The present method can be applied in forensic casework, including those with limited DNA quantity and quality. In the present study we evaluated a powerful tool for identification. DNA analysis looked at different areas of the genome with genetic evaluation which can be very accurate in detecting human eye's color space. SNP involved in external traits creating a new route using genetic research and criminal investigations. In recent years, significant progress has been observed in the field of SNP detection engaged in external phenotypic. In the elongation of a primer (SBE) based on the accuracy of the enzyme, DNA polymerase is very reliable. SNapShot based technologies are now common in the forensic laboratory and the most important is the capillary electrophoresis device.

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