Original Article

Evaluate 11 Plex SNPs of the MC1R Gene for Eye Color Prediction using the SNaPshot Technique in an Iranian Population

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Abstract

The most ambitious DNA phenotyping goal is forecasting a full face from a DNA sample. Over the last decade, GWAS and successive predictive analyses have discovered a multitude of EVC-predictive SNPs and predictive models, most particularly for human pigmentation features. IrisPlex, the first forensic eye color prediction system, was designed mainly to differentiate between blue and brown eyes, and its evolved version, the HirisPlex-S DNA testing method, predicts eye, hair, and skin color based on DNA traces. 59 DNA samples were examined using the Multiplex SnaPshot kit (Applied Biosystems) for the simultaneous detection of 11 SNPs (rs1805005, rs885479, rs11547464, rs185008, rs1805006, rs1805007, rs1805009, rs2228479, rs1110400) taken from a large-scale GWAS research. This study adopted the genotype identification of 11 Hirisplex system markers as a prediction model of eye color and hair color. Some of these variants (rs11547464, rs885479, rs1805005, rs2228479) were found to be relevant for anticipating eye and hair color, whereas others (rs1805008, rs1805006, rs1805007, rs1805009, rs1110400, Y1520CH, N29insA) were observed to be unacceptable because of low variation. The statistical data illustrated a substantial level of agreement between the statistical model and the actual eye color of the participants.

Keywords: Hair color, Eye color, MC1R, SNaPshot, Iranian Population

Introduction

Forensic DNA phenotyping (FDP) uses basic genetic knowledge to understand more about an unknown crime scene donor. FDP is being introduced into the field of forensic genetics using large-scale sequencing methods for two reasons. First, it overcomes the limitations of conventional

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human identification techniques, and second, it is evidently advantageous as a police investigative tool to narrow the group of suspects[1]. DNA phenotype allows the projection of phenotypic traits based on genetic information. In cases where there is no proof to identify a person, this reduces the number of likely suspects. The forensic community's interest in phenotypic trait prediction has grown as more genes associated with various physical traits have been discovered[1,2]. Most human traits are considered complex because they are influenced by multiple genes as well as environmental factors, and their variability is continuous (Serrano, 2020). Eye, hair, and skin pigmentation are among the most studied human phenotypic traits, as well as facial features, since they provide the most data in criminal cases when searching for and identifying suspects. Because the former phenotypic traits are less complicated, studies on the genetics of human pigmentation are more sophisticated than studies on facial traits. This is because they have semi-Mendelian heredity, meaning that a small number of genes provide most of the phenotypic information [1,3]. Pleiotropic effects, in which a single SNP affects more than one phenotypic trait, accelerate the genetic difficulty of color[4]. The same is true for the occurrence of epistasis, which takes place when many SNPs interact in the creation of a single trait[1]. SNPs are single nucleotide polymorphism markers that regulate the phenotype of an individual. They have now been demonstrated to be very suitable alternative markers for STRs in criminological studies[5]. SNPs have several benefits over STRs in forensic studies, including a lower mutation rate, which is critical in kinship testing. Another benefit of SNPs is that they can be examined quickly, comprehensively, and automatically[6]. Today, attempts are being made to study more than 50 SNPs simultaneously in a short period of time. Although several research efforts have been conducted to evaluate the viability of using SNPs in different contexts, no unified approach for using SNPs in criminal investigations has been offered[7].

The most ambitious aim of DNA phenotyping is to predict an entire face based on a DNA sample[8]. Several attempts have been made to develop a predictive face model [8–11]. GWAS and subsequent predictive analyses have found several EVC-predictive SNPs and predictive models over the past decade, notably for human pigmentation traits[9]. The first forensic eye color prediction system, IrisPlex [12] was explicitly developed to distinguish blue and brown eyes[13]. The HIrisPlex-S prediction system is the latest test that can predict eye, hair, and skin color simultaneously [1,13]. HIrisPlex-S DNA testing technology enables simultaneous projection of eye, hair, and skin color based on DNA traces. The FDP system consists of two SNaPshot-based multiplex assays targeting a total of 41 SNPs, including 24 SNPs for eye and hair color prediction and 17 SNPs for skin color prediction [9]. Walsh et al. conducted a study on 6168 Dutch populations to confirm the HIrisPlex system. The outcomes of this study showed that some variations of the melanocortin-1 receptor gene (MC1R) have high penetrance [12]. In the study conducted by Spichenok et al. on 544 individuals, they concluded that the iris plex system has a low error rate in determining eye color in different races in the United States. In fact, this study confirmed the accuracy and correctness of this marker collection [14]. Allwood et al. and Kastelic et al. also confirmed the capability of the iris plex system to predict eye color [15(p2013)]. (In order to provide forensic intelligence SNP data from latent DNA, Young et al. report the first use of direct PCR combined with MPS by examining the HIrisPlex System [16]. Breslin et al. provide massively parallel sequencing (MPS) possibilities for the HIrisPlex-S (HPS) system using the two MPS technologies frequently used in forensics, Ion Torrent and MiSeq, to cover all 41 DNA variants in a single test. They also exhibit the forensic developmental validation of the two HPS-MPS tests[17].

In this field, many similar experiments have been conducted worldwide, but in Iran, such a study based on the allele frequency of SNPs of the HIrisPlex system has not been conducted yet. The objective of this investigation was to investigate the frequency of some SNP markers (1805005, rs885479, rs11547464, rs185008, rs1805006, rs1805007, rs1805009, rs2228479, rs1110400) related to hair and eye color in a sample of Iranians.

Materials and Methods

Blood samples were randomly collected from 59 subjects, including 22 men and 37 women of different Iranian ethnicities, among unrelated individuals; DNA was then extracted from all samples using a

standard salting-out protocol. Multiplex design and genotyping methodology A multiplex single-base extension reaction test was utilized to examine 59 DNA samples. All participants were tested for 11 SNPs simultaneously. We developed a multiplex single-base extension approach for the simultaneous identification of every 11 SNPs (rs1805005, rs885479, rs11547464, rs185008, rs1805006, rs1805007, rs1805009, rs2228479, rs1110400) taken from a large-scale GWAS research (Liu et al., 2010), employing the Multiplex SNaPshot kit (Applied Biosystems) for the reaction, followed by capillary electrophoresis on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). The PCR primers were designed with OLIGO Primer Analysis Software Version 7 and Gene Runner Version 6.5.52x64 Beta to generate PCR fragments ranging from 106 to 158 bp. To eliminate primer-primer interactions, all primer sequences were analyzed using the Auto Dimer software application [47] before laboratory analysis. Table 1 contains all of the primer sequences. All 11 SNPs were amplified using PCR primers (5 pmol concentration), 1 µl genomic DNA (50 ng concentrations), and attempts to use master mix Red2x (AMPLIQON) in a single multiplex PCR assay in a total volume of 25 µl.

Table 1: The quantity of AUCs estimated for the hair and eye color SNPs in the research

	Hair color			Eye color	
SNP	**Black Hair**	**Brown Hair**	**Red Hair**	**Intermediate eye**	**Blue eye**
rs11547464	0.532	0.658	0.636	0.531	0.531
rs885479	0.524	0.505	0.532	0.542	0.542
rs1805005	0.526	0.608	0.638	0.510	0.510
rs2228479	0.559	0.540	0.503	0.522	0.522

All amplifications were performed on a Minicamp™ Plus Thermal Cycler (Thermo Fisher scientific) with the following conditions: 95°C for 10 minutes, 35 cycles of 94°C for 1 minute, 64.4°C for 1 minute, and 72°C for 1 minute. The last extension was 10 minutes at 72 degrees Celsius. The amplified PCR products were purified using EXO I (exonuclease I) and SAP (shrimp alkaline phosphatase) and incubated for 60 minutes at 37°C and 15 minutes at 75°C. In the following single-base extension technique, the SBE primers annealed around the single-nucleotide polymorphism (SNP) region. Poly (dt) tails of varying lengths were added to single-base extension primers (Table 2).

Table 2: Sequences of Single-Base Extension Reaction Primers and PCR Primers

SNP	primer	Sequence (5′-3′)
rs12913832	PCR For	TAATTCAAAATGCCCCCAAG
	PCR Rev	TGATAGCGTGCAGAACTTGACA
	SNaPshot	TGATGATAGCGTGCAGAACTTGACA
rs1800407	PCR For	TGAAAGGCTGCCTCTGTTCT
	PCR Rev	TATGGTCACAGGCGTGAAGA
	SNaPshot	GACTGACTGACTGACTGACTGACTGACTGACGTGGCCAGGCATACC GGCTCTCCC
rs1393350	PCR For	GATGCGTGCATATCCACCAACT
	PCR Rev	GGTGAATGATAACACGAACAGATT
	SNaPshot	GACTGACTGACTGACTGACTGACTGACTGAGTAAAAGACCACACAGATTT
rs16891982	PCR For	GGAAAACACGGAGTTGATGC
	PCR Rev	CCAGAGGTGGAGAAGCAGAG
	SNaPshot	GACTGACTGACTGACTAGAGGAGTCGAGGTTGGATGTTGGGGCTT

Cont....

rs12896399	PCR For	CTGGCGATCCAATTCTTTGT
	PCR Rev	GACCCTGTGTGAGACCCAGT
	SNaPshot	ttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttTCTTTAGGTCAGTATATTTTGGG
rs12203592	PCR For	AGGGCAGCTGATCTCTTCAG
	PCR Rev	GCTTCGTCATATGGCTAAACCT
	SNaPshot	ttttttttttttttttttttttttttttttttttttttttttttttttTCCACTTTGGTGGGTAAAAGAAGG

The SNaPshot reaction contained 1 µL of primer mix, including all 11 single-base extension primers (final concentration of 1 pmol each), 1.5 µL of purified PCR products, and 2.5 µL of SNaPshot Multiplex kit (Applied Biosystems by Thermo Fisher Scientific) in a large volume of 5 µL. The PCR condition consisted of 25 cycles of 10 seconds at 95°C, 5 seconds at 50°C, and 30 seconds at 60°C. After the reaction, the 5′-phosphoryl groups of unincorporated dideoxy nucleotide triphosphates were removed by adding 1.0 U of CIP (Calf Intestine Phosphatase) and incubating the enzyme at 37°C for 45 minutes and 75°C for 15 minutes. Capillary electrophoresis was carried out using an ABI PRISM 3130 Genetic Analyzer, and for allele calling and data analysis, Gene Mapper ID, Ver. 3.7 (Thermo Fisher Scientific) was used. A number of samples were submitted for sequencing to confirm the observations of the SNaPshot approach from all 11 SNPs. Iris Photography Tocompares and assess, the iris of all participants was photographed in two ways. Due to the sensitivity of the study and inclusion criteria, the expert physician was supported in identifying the samples that did not fulfill the inclusion condition in the first technique. The second method was to examine HD-quality photographs of the iris of study participants taken using a digital camera and the same photographic conditions. The aim of iris photography was to discover a reasonable and effective link between each individual's iris color and their IrisPlex profile in the study population. Topcon camera photography (by an ophthalmologist) After validating the inclusion criteria, all participants were evaluated by a specialist who photographed their iris. It should be mentioned that the ophthalmologist investigated the natural color of the iris, illnesses, injuries, and the application of effective medications in the color of all the volunteers' eyes. We used a Topcon IM 900 camera (Fig. 1) and EyeSuite software to process the photographs. To analyze the iris with Eyesite software, we needed standard and high-quality photographs taken in standard conditions of the volunteer's eyes. The Canon EOS 70D professional camera with the Canon EF 100mm Macro F2.8 L IS USM lens was used for all photography in similar conditions in terms of distance and amount of light. We also developed software to identify eye color using IMI and RGB indices in partnership with the Department of Biomedical Engineering at Islamic Azad University.

Fig 1: The spectrum of different decreasing colors of the iris from 1 to 12, photographed with a Canon PowerShot digital camera and a macro lens by a professional photographer under the identical environmental conditions for all participants

inquiry into hair color

The red hair color was assessed using people's self-reports, family history, and the completion of a questionnaire. The photograph was shot with a Topcon camera at a distance of 7 cm from the back of the head to examine the color of the hair.

In this study, we employed a multi-nominal regression model and the HIrisPlex system's resultant profile to reliably predict blue and intermediate eye color, as well as black, brown, and red hair color.

Results

The multiplex SNaPshot approach was confirmed by assessing 10 DNA samples genotyped earlier by DNA sequencing for each examined SNP. To ensure the performance of SNaPshot (Fig. 2) from each series of samples associated with an SNP detected by the SNaPshot diagnostic system, we sequenced several samples, and the sequencing findings validated the SNaPshot results (Fig. 3).

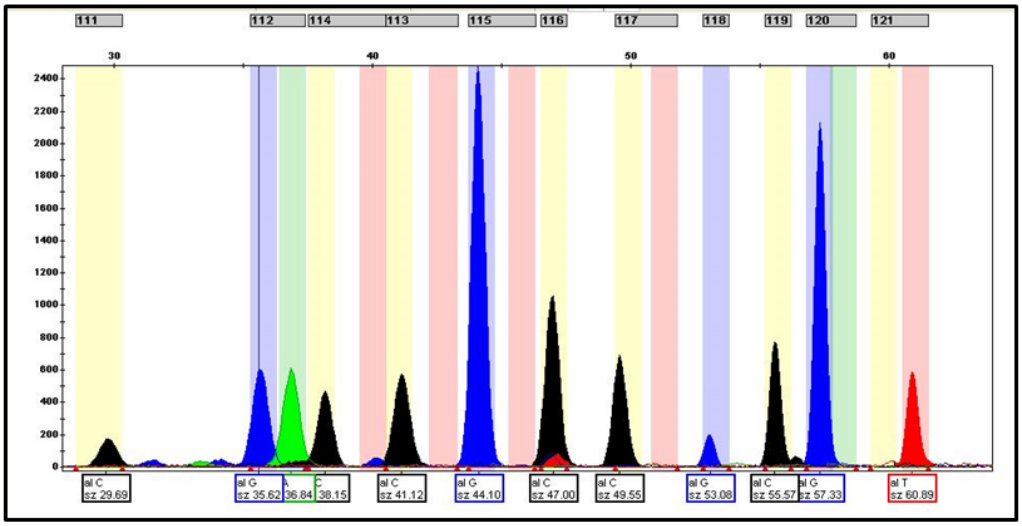

Fig 2: The SNaPshot Profile of a person with red hair. Each peak corresponds to a single SNP: 111(N29insA), 112 (rs11547464), 113(rs885479), 114(rs1805008), 115(rs1805005), 116(rs1805006), 117(rs1805007), 118(rs1805009), 119(Y152OCH), 120(rs2228479), and 121(rs1110400).

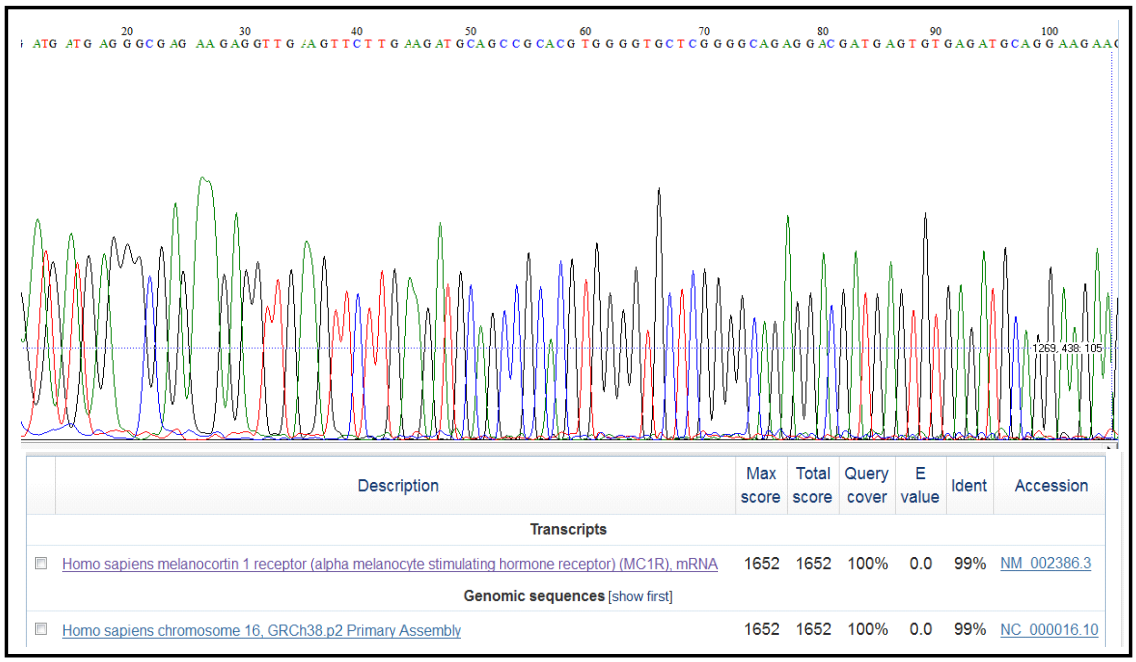

Fig 3: The match is significant since the E-value is zero.

In this study, genotype identification of 11 HIrisPlex system markers [12]. was employed as a prediction model of eye color and hair color for each of the 59 samples. Statistical research revealed that certain variations (rs11547464, rs885479, rs1805005, rs2228479) were acceptable for predicting eye and hair color, but others (rs1805008, rs1805006, rs1805007, rs1805009, rs1110400, Y1520CH, N29insA) were unacceptable owing to low variation. The probability of having any of the eye colors (blue and intermediate)

and any of the hair colors (black, brown, and red) has been employed in prior research through authorized and published multinomial logistic regression models [3,18]. The ROC curve was utilized to enhance the performance of predictive models, including the area under the ROC curve (AUC), which shows the value of some sensitivity and specificity of the eye and hair color prediction model (Fig. 4). The determined AUC for our markers is shown in Table 1. The statistical findings revealed a significant degree of agreement between the statistical model and the participant's real eye color.

Discussion

Human hair color is one of the most noticeable characteristics. Twin studies estimate that heritable elements may explain up to 97% of the difference in hair color, and genome-wide association studies (GWAS) have found various chromosomal regions associated with hair color and other pigmentation traits [9]. Eye color is a highly polymorphic trait that has been proven to be multigenic in the European population by genome-wide association studies [18]. The accuracy of the HIrisPlex systems SNP markers in the diagnosis and projection of eye and hair color in the European population are now well established [12,17,19]due to the impending ability to predict externally visible characteristics (EVCs. The outcomes of the HIrisPlex system assessment in European populations with large sample sizes demonstrate that the alleles associated with blue and brown eye color correspond to the parameters of the logistic regression model [12]due to the impending ability to predict externally visible characteristics (EVCs. The logistic regression model has an adequate value for predicting blue and brown eye color; however, it was less valid for intermediate eye colors [20]. The HIrisPlex system is intended to study tiny quantities of DNA material, including damaged DNA. It is employed in forensic genetics for this reason, and for populations of European origin, its prediction has proven reliable. A thorough evaluation of its accuracy for people from other nations has not been made [7]as well as in studies of ancient human populations. However, the accuracy of this tool has been verified on the West and Central Europeans only, while populations from border regions between Europe and Asia (like Caucasus and Ural. We assessed the HIrisPlex system in a target population of Iranians for the first time in this study, which was verified and analyzed to reliably detect eye color and hair color owing to the presence of numerous ethnicities and races in the Iranian community. These findings are consistent with studies conducted in previous years. Hair color prediction and categorization are more prone to mistakes because elements such as darkness and intensity, as well as environmental factors, particularly lifespan, influence hair color variations. The most popular age-related hair color changes are from bright blonde in childhood to dark blonde and bright or dark brown in maturity. This might be described by hormonal changes throughout puberty. However, its chemical basis is unknown at this time. The HIrisPlex system is unable to differentiate between these variations in individuals and the stability of hair color from childhood to adulthood. Some variants on the MCR1 gene consist of N29insA, rs11547464, rs1805005, rs1805006, rs1805007, rs1805009, and Y152OCH, and rs1805009 are very important variations in Walsh's study, while the variants rs885479, rs1805005, and rs2228479 indicate a varied distribution in the European population and its nearby areas. These polymorphisms of the MC1R gene were explored in research done in a chosen population of Tehran, and the results were verified in persons with red hair. We employed a statistical logistic regression model in this investigation to properly predict eye color. We predicted the precise color of blue, brown, and intermediate colors using the HIrisPlex model. In this model, the correct prediction rate (AUC) for blue eyes was 0.66, for the intermediate color 0.66, for black hair 0.68, for brown hair 0.68, and 0.82 for red hair. The sensitivity level for intermediate eye color is 100%, whereas the sensitivity level for blue eye color is 0%, indicating that these indicators are ineffective for predicting blue eye color, and it cannot tell the difference between blue and intermediate eye colors. And the specificity for blue eyes is 100%, whereas the specificity for intermediate eyes is 0%. In addition, the sensitivity of this model is 91% for black hair, 94.3% for brown hair, and 63.6% for red hair. Furthermore, the application of these SNP markers facilitates the categorization of eye color; nevertheless, little research has been undertaken with this scheme in the classification of eye color so far [19]. The optimum concentration of DNA in the SNaPshot multiplex

reaction for eleven HIrisPlex system markers was between 0.3 and 0.5 ng, but for the AmpF/STR Minifiler identification test for eight STRs, we require 125 pg. In reality, the HIrisPlex system has a far higher sensitivity than the AmpF/STR Minifiler [21]. Because the sensitivity of SNP testing is expected to be higher than that of STR tests, the combined system of the SNP/STR multiplex is proposed in forensic investigations owing to the significant significance of the subject [12]due to the impending ability to predict externally visible characteristics (EVCs. In conclusion, to gain an improved understanding of the high-scale research of SNP markers and their capacity to predict EVCs in individuals with mixed genetic backgrounds, we need to examine a variety of populations with different genetic backgrounds and increase the sample size. Furthermore, additional SNP markers are required to discriminate eye colors, notably intermediate colors, with confidence.

Conclusion

Predicting an entire face from a DNA sample is the most demanding DNA phenotyping challenge[11]. Several EVC-predictive SNPs and predictive models have been found during the past ten years through GWAS and subsequent predictive analysis, most notably for human pigmentation traits [20(p2018)]. The first forensic eye color prediction system, IrisPlex [17], was created primarily to distinguish between blue and brown eyes. Its upgraded version, the HirisPlex-S DNA testing technique, uses DNA traces to predict eye, hair, and skin color [1]. In this investigation, the Multiplex SNaPshot kit was utilized to simultaneously identify 11 SNPs that were obtained from a large-scale GWAS study for 59 DNA samples belonging to an Iranian population. A model for predicting eye color and hair color was developed using the genotype identification of 11 markers from the HIrisPlex system. Only some of these variations, according to the results, are suitable for predicting eye and hair color, while others are unsuitable owing to limited variation. The statistical data showed that the statistical model and the individuals' real eye colors agreed to a significant extent. Due to the diversity of nationalities and races within Iranian society, we evaluated the HIrisPlex system in this study's target group of Iranians for the first time. The system was confirmed and examined to precisely determine eye color and hair color. These results are by research from earlier years. In closing, we need to explore a range of populations with varied genetic origins and expand the sample size to better understand the high-scale research of SNP markers and their ability to predict EVCs in individuals with mixed genetic backgrounds. To confidently distinguish between eye colors, especially intermediate colors, additional SNP markers are needed.

There are no conflicts of interest to declare.

The study was conducted in accordance with ethical standards, and informed consent was obtained from all participants.

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