THE TWO SINGLE NUCLEOTIDE POLYMORPHISM HAPLOTYPES ON CHROMOSOME 15 OF THE HERC2 AND OCA2 GENES OF THE COLOR VARIATION OF THE HUMAN EYE IN A SAMPLE OF IRAQI POPULATION

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ABSTRACT

This study was aimed to identify the role of the single nucleotide polymorphism genes on the contrast of three groups (Dark-Brown, hazel and blue) of human eye color among a sample of Iraqi people specifically single nucleotide polymorphism (SNPs); the *rs12913832* of HERC2 gene and the *rs1800407* of OCA2 gene, for 90 human blood samples of different people were collected from College of Science, University of Baghdad and also people from different areas of Baghdad, Iraq. These SNPs were analyzed using quantitative real-time PCR (*q*PCR). The results of the *rs12913832* and *rs1800407* genotype showed significant differences (*p <0.05*) in the blue eye color groups. the *rs12913832* of *HERC2*; *rs1800407* of the *OCA2* genotypes and allele frequencies in contrast to the dark brown and blue iris group showed that there was significant variation in the heterozygous genotype frequencies, while these SNPs in contrast to the dark brown and hazel iris group showed a non-significant effect in a sample of Iraqi population. The haplotype statistical analysis shows strongly Linkage disequilibrium (LD) between the *rs*12913832 of HERC2 gene and the *rs*1800407 of OCA2 gene in dark-brown, hazel and blue eye color subjects.

Keywords: **iris, allele frequencies, haplotype, linkage disequilibrium (LD).**

مجلة العلوم الزراعية العراقية -2022 :53 (1):67-74 **نعمان و القزاز**

للتباين اللوني للعين البشرية في عينة النوعان المفردان من النيوكليوتيدات المتعددة الأشكال على الكروموسوم 15 من جينات OCA2 و HERC2 من السكان العراقيين

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المستخلص

هدفت الدراسة الى التعرف على دور تعدد أشكال النوكليوتيدات المفردة *rs12913832* لجين HERC2 و *rs1800407* للجين OCA2 بالمقارنة من ثلاث مجموعات (بني داكن ، عسلي ، أزرق) من لون عين الإنسان بين عينة من الشعب العراقي ، تم جمع 90 عينة دم بشرية من أشخاص مختلفين من كلية العلوم ، جامعة بغداد وأيضًا أشخاص من مناطق مختلفة من بغداد ، العراق. تم تحليل SNPs باستعمال PCR real time الكمي (qPCR). أظهرت نتائج التركيب الوراثي *rs12913832* و *rs1800407* اختلافات معنوية (p <0.05) في مجموعة لون العين الزرقاء. واظهرت *rs12913832* من جين HERC2 ؛ *rs1800407* من جين *OCA2* للأنماط الجينية وترددات الأليل بالمقارنة من مجموعة القزحية ذات اللون البني الداكن والأزرق تباينًا كبيرًا في ترددات النمط الجيني متغاير الزيجوت ، بينما أظهرت هذه SNPs بالمقارنة من مجموعةالقزحية البني الداكن والعسلي ، تأثيرًا غير مهم بين عينة من الشعب العراقي. اظهر التحليل الإحصائي للنمط الفرداني بشدة عدم توازن الارتباط (LD) بين *rs12913832* لجين HERC2 و *rs1800407* لجين OCA2 في الموضوعات ذات اللون البني الداكن والعسلي واللون الأزرق..

كلمات مفتاحية: قزحية, ترددات الاليل, النمط الفرداني, اختلال التوازن (LD).

Received:22/12/2020, Accepted:1/3/2021

INTRODUCTION

The pigmentation of the human iris is mainly determined by the pigment melanin, which varies from yellow to black depending on the type of melanin (14). In humans, it is the main determinant of pigmentation of the skin, hair, and iris. Eye color has a high degree of heritability, ensuring that the variation in the human genome explains the difference in this phenotype. Many polymorphs are thought to play a role in determining eye color (4). The vast majority of iris pigment heterogeneity undergoes a mutation in a phenotype (single nucleotide polymorphism) called *rs12913832* (6, 17). This marker is thought to be responsible for determining the distinction between blue and brown eyes found in the intron 86 of the HERC2 gene. The mononucleotide polymorphism (SNP) may carry an allele for guanine or adenine. The guanine allele is present in the European groups of very high frequencies and is associated with blue irises. The allele for adenine is present in clusters of non-European origin at very high frequencies and is associated with a brown iris. This SNP works in a dominant / recessive manner. People with a homozygous guanine allele have a blue iris, but homozygous or heterozygous people with a homozygous guanine allele have a brown iris. One study group found that individuals with the G / G genotype only had a 1% chance of developing a brown iris, but those with the A / A genotype had an 80% chance of developing a brown iris. Comparative research shows that 98.3% of people with the A / A genotype have an 80% chance of staining, and suffer from a blue iris is homozygous for the generated allele (9; 16). The OCA2 expression in individuals with *rs12913832:* G which has melanocytes that are light- pigmented was found to be decreased in comparison with those in individuals with *rs12913832:* A, which have melanocytes that are darkly pigmented. According to the predominant theory, individuals with brown eyes were the outcome of persons with the rs12913832: AA or the *rs12913832*: GA genotype. However, this phenomenon was often not the case for individuals with the *rs12913832:* GA genotype, which can result in intermediate or blue eyes (10). The OCA2 study is performed subsequently in conjunction with the eye color, but common variants contribute to eye color variations. Single-nucleotide polymorphisms (SNPs) are first engaged in the inheritance of the eye color variation in Europeans in the molecular area of OCA2 (18, 19) The SNP *rs12913832* of the HERC2 gene may play the primary role in regulating the difference between brown and blue irises, and the complete incompatibility between HERC2 and iris pigmentation suggests that many other markers may be involved. During 2009, iris color was predicted in the Netherlands using DNA markers. They found six polymorphs that act as major genetic markers of eye color in six different shapes. The HERC2 genes include *rs12913832, OCA2 rs1800407,* and other SNPs. Moreover, an OCA2 coding SNP (*rs1800407*) acts as a penetration modifier for *HERC2 rs12913832* SNP for eye color (4; 11). The *rs1800407* of the OCA2 gene has little frequency but has good effects on eye color in all human species. This SNP is considered the second best indicator of eye color diversity in Europeans (9; 19). A major genetic determinant of blue, medium, and brown eye color was developed in the *OCA2 / HERC2* region. Several studies describe polymorphism, which may be directly responsible for the differences in pigmentation of the iris (3, 7, 17) .The present study aims to find out the relationship between *rs12913832* and *rs1800407* SNPs and their direct relationship to eye color.

MATERIALS AND METHODS

Specimens' collection

The Ethics Committee has accepted this study, with the reference number (CSEC/0121/0009) in Department of Biotechnology, College of Science, University of Baghdad; Baghdad, Iraq. The sample groups consist of adult healthy males and females over 18 years old, involved 90 unrelated individuals from Baghdad. Samples were divided to three groups a dark-brown eye color (X), hazel eye color (Y) and blue eye color (Z).

Single nucleotide polymorphisms of (OCA2 and HERC2) genes

Designed primers and probes for OCA2 gene (*rs1800407* G>A); HERC2 gene (*rs12913832* A>G), they provided in a lyophilized state by Alpha-DNA Company (Canada) stored at (-

20°C). The sequences **of** each of the probes and primers used in the allelic discrimination experiments are show in Table 1. ReliaPrep™ Blood gDNA Mini-prep System (Promega, Canada) was used to extract genomic DNA from leukocytes of the blood samples according to the manufacturer instructions. Genotypes were detected by *Taq*-Man allelic discrimination assay on a quantitative real-time thermo-cycler (Qiagen Rotor Gene, Germany). The components of the amplification reaction and their final concentrations are show in Table 2, qPCR was programmed after the optimization.

Table 1. Designed Probes and Primers of HERC2 (*rs12913832*) and OCA2 (*rs1800407*) genes (Alpha-DNA, Canada).

Gene name and SNP		Primers and probes 5́ ⟶ 3́
HERC2	Forward	TTGTTCTTCATGGCTCTCTGTG
	Reverse	CTCGGCCCCTGATGATGATA
rs12913832	Wild-probe	TGAGCATTAAATGTCAAGTTCTGCA
	mutant-probe	TTTGAGCATTAACTGTCAAGTTCTG
OCA2	Forward	TGGTGACGTTGTCCAAGAAG
	Reverse	TGGCTTGTACTCTCTCTGTGT
rs1800407	wild-probe	TCCCGGGGAGAGCCG
	mutant-probe	CCGTCCCAGGGAGAGC

Table 2. The components of the amplification reaction

qPCR Components		Volume (µl)
Master Mix		10
Primer	Forward	0.5
	Reverse	0.5
Probe/Wild		0.5
Probe/Mutant		0.5
Nuclease-Free Water		3
DNA		5
Total volume		20µl

Statistical analysis

The Hardy-Weinberg equilibrium (HWE) was applied to study the number of genotype and allele frequencies (5, 13). The odds ratios (ORs) and confidence intervals (95% CIs) were used to determine the potential associations between genetic SNPs of HERC2 and OCA2 genes of eye color all groups in this study by computer program (win pepi version11.65). Statistical significance was considered as a p-value < 0.05 (5;14). The SHEsis software (http://analysis.bio-x.cn) was used to haplotype SNPs, which illustrate the linkage disequilibrium (LD) between polymorphisms of HERC2 and OCA2 genes (20).

RESULTS AND DISCUSSION

Distribution of subjects according to the gender: All these samples were collected from healthy individuals with a ratio of about (59.25%) male and (40.75%) female ; Fig. 1 represent the gender ratio of the samples for each group; with age 18 years and above. In addition, some pictures were taken by using High-resolution shots at ultra/Micro-close (Nikon D750) shown in Fig.2 to represent the phenotypes of pigmentation in each group

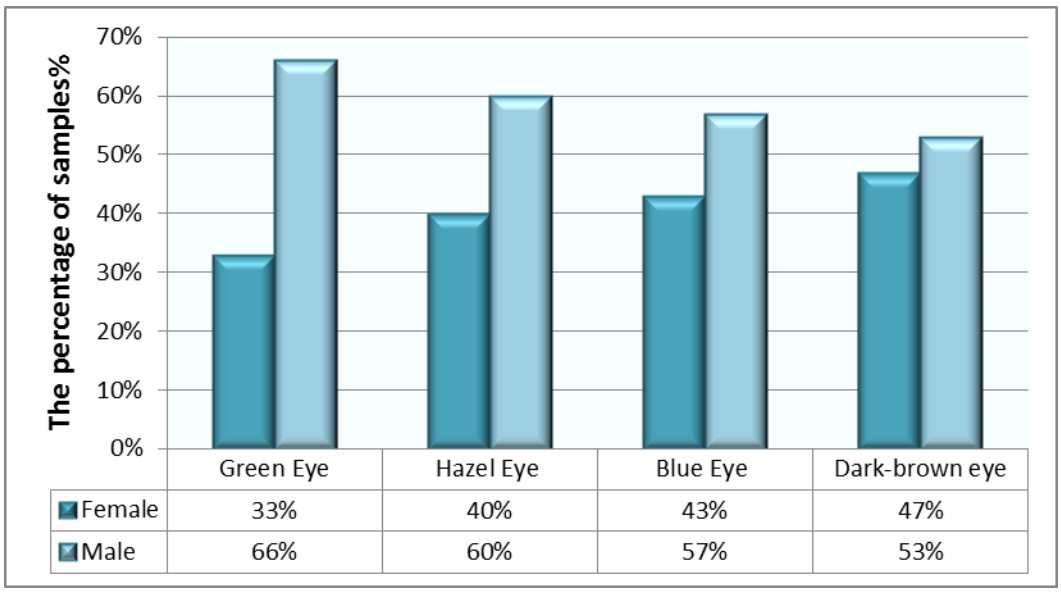

Figure 1. Percentage of blood samples

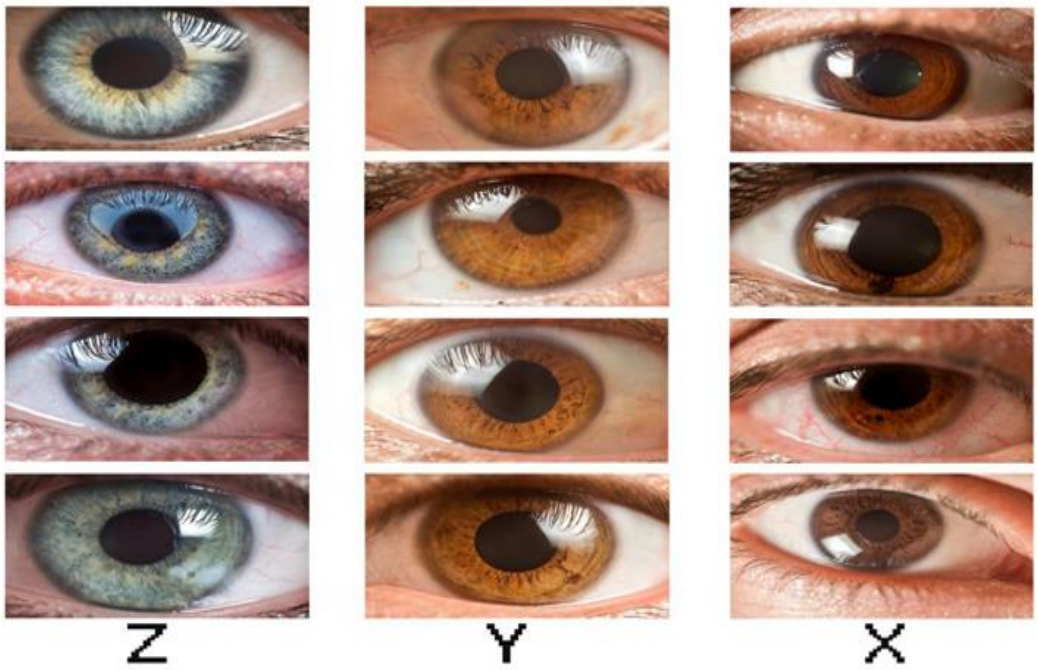

Figure 2. Eye color image of 12 unrelated individuals. (X) Dark-brown eye color. (Y) Hazel eye color. (Z) Blue eye color. The image was taken directly High-resolution shots at ultra/Micro-close (Nikon D750).

Genotyping of HERC2 (*rs*12913832) and OCA2 (*rs1800407*) genes

The SNP of the HERC2 gene (A > G; *rs*12913832) was presented with three genotypes (AA, AG, GG) and two alleles (A and G). Analysis of Hardy-Weinberg equilibrium (HWE) in (X) and (Z) groups revealed that the genotypes were consistent with equilibrium, and non-significant differences (p <0.05) and Hardy-Weinberg equilibrium (HWE) in the (Y) group revealed that the genotypes were not consistent with equilibrium, a significant differences (p =0.003) were detected between the observed and expected genotype frequencies in both groups (Table 3).The SNP of the OCA2 gene (G > A; *rs*1800407) was presented with three genotypes (GG, GA, AA) and two alleles (G and A). Analysis of Hardy-Weinberg equilibrium (HWE) in (X), (Y) and (Z) revealed that the genotypes did not consistent with equilibrium, and significant differences (p <0.05) were detected between the observed and expected genotype frequencies in both groups (Table 3).

Table 3. Number and percentage frequencies of *rs12913832* and *rs1800407* genotypes and their Hardy-Weinberg equilibrium (HWE) in (X), (Y) and (Z) groups

Variant	Geno-type	Dark-Brown eyes (X) group				Hazel eyes (Y) group				Blue eyes (Z) group			
		Observed		Expected		Observed		Expected		Observed		Expected	
		n	%	n	%	n	%	n	%	n	%	n	%
HERC2 *rs12913832*	AA	18	60.0	19.2	64	21	70.0	21.67	72.25	9	30.0	8	26.69
	AG	12	40.0	9.6	32	9	30.0	7.65	25.5	16	53.4	14.98	49.94
	GG	0	0.0	1.2	4	0	-	0.68	2.25	5	16.6	7	23.36
HWE Analysis		P-value = 0.171 Non- Significant				P-value = 0.003 Significant				P-value = 0.334 Non- Significant			
OCA2 *rs1800407*	GG	12	40.0	14.7	49	10	33.3	12.68	42.25	5	16.6	10.21	34.03
	GA	18	60.0	12.6	42	19	63.3	13.65	45.5	25	83.4	14.58	48.61
	AA	0	-	2.7	9	1	3.3	3.67	12.25	0	-	5.21	17.36
HWE Analysis		P-value = 0.019 Significant				P-value = 0.032 Significant				P-value < 0.001 Significant			

Inspecting HERC2 gene genotypes and allele frequencies in dark-brown *vs.* hazel (H) group revealed that there was non-significant variation between these frequencies, although increased frequencies of the A allele (80 *vs.* 85.0%) and decreased frequencies of G allele (20 *vs.* 15.0 %) were observed in hazel eye color (Y) compared to dark-brown eye color (X) (Table 4). In AG Polymorphism, the odds ratio for the AG genotype was 0.64 with *p*=0.589 indicating that non-significant of AG and of (H) group (Table 4). Inspecting HERC2 gene genotypes and allele frequencies in dark-brown vs. blue (R) group revealed significant variations between these frequencies, although decreased frequencies of the A allele (80 *vs.* 56.6%) and increased frequencies of G allele (20 *vs.* 43.4 %) were observed in blue eye color (Z) compared to dark-brown eye color (X) (Table 4). In AG Polymorphism, the odds ratio for the AG genotype was 4.93 with *P*-value = 0.008 indicating that significant of AG of (R) group and the odd ratio of G allele was 3.74; so the *P*-value = 0.002 indicating that significant of G allele of (R) group, (Table 4).

Table 4. Genotype and allele frequencies of *rs12913832* SNP of dark-brown vs. hazel eye color (H) group and dark-brown vs. blue eye color (R) group of blood samples

Type of Comparison	*rs12913832*	Control N	Control %	Case N	Case %	P-value	OR(95% CI)
Dark- Brown *vs.* Hazel eyes (H) group	AA	18	60.0	21	70.0	-	1.00(Reference)
	AG	12	40.0	9	30.0	0.589	0.64 (0.22 to 1.84)
	GG	0	-	0	-	-	-
	A	48	80.0	51	85.0	-	1.00(Reference)
	G	12	20.0	9	15.0	0.632	0.71 (0.28 to 1.81)
Dark- Brown *vs.* Blue eyes (R) group	AA	18	60.0	4	30.0	-	1.00(Reference)
	AG	12	40.0	23	53.4	0.008	4.93 (1.64 to 14.79)
	GG	0	-	3	16.6	0.237	7.76 (0.40 -149.64)
	A	48	80.0	31	56.6	-	1.00(Reference)
	G	12	20.0	29	43.4	0.002	3.74 (1.68 to 8.36)

Inspecting OCA2 gene, genotypes and allele frequencies in dark-brown vs. hazel (H) group revealed that there was non-significant variation between these frequencies, although decreased frequencies of the A allele (70.0 *vs.* 65.0%) and increased frequencies of G allele (30 *vs.* 35.0 %) were observed in hazel eye color (Y) compared to dark- brown eye color (X) (Table 4). In AG Polymorphism, the odds ratio for the AG was 1.15 with P-value = 0.546 indicating that non-significant of AG and of (H) group (Table 4). Inspecting HERC2 gene, genotypes and allele frequencies in dark-brown vs. blue (R) group revealed that there was significant variation between these frequencies, although decreased frequencies of the A allele (70 *vs.* 58.3%) and increased frequencies of G allele (30 *vs.* 41.7 %) were observed in blue eye color (Z) compared to dark-brown eye color (X) (Table 4) In AG Polymorphism, the odds ratio for the AG genotype was 3.33 with *P*-value = 0.045 indicating that significant of AG of (R) group, (Table 4). Analysis of haplotypes between

alleles of two investigated SNPs revealed strong linkage disequilibrium (LD) as defined by the estimated LD coefficient (D') illustrate in (Table 5) and correlation coefficient (r^2). The LD was shown in Fig.3 Linkage disequilibrium coefficient between *rs*12913832 and *rs*1800407, the D' of dark-brown eye (X) was 0.69, hazel eye (Y) was 0.98 and blue eye (Z) was 0.80.

Table 4.Genotype and allele frequencies of *rs1800407* SNP of dark-brown vs. hazel eye color (H) group and dark-brown *vs*. blue eye color (R) group of blood samples

Type of Comparison	*rs1800407*	Control N	Control %	Case N	Case %	P-value	OR(95% CI)
Dark- Brown vs. Hazel eyes (H)	GG	12	40.0	10	33.3	-	1.00(Reference)
	GA	18	60.0	19	63.3	0.546	1.15 (0.41~3.21)
	AA	0	0.0	1	3.3	1.00	1.00(Reference)
	G	42	70.0	39	65	-	1.00(Reference)
	A	18	30.0	21	35	0.558	1.26 (0.58~2.70)
Dark- Brown vs. Blue eyes (R)	GG	12	40.0	5	16.6	-	1.00(Reference)
	GA	18	60.0	25	83.4	0.045	3.33 (1.02~0.91)
	AA	0	-	0	-	-	-
	G	42	70.0	35	58.3	-	1.00(Reference)
	A	18	30.0	25	41.7	0.183	1.67 (0.79- 3.52)

Table 5. Impact of *rs12913832* and *rs1800407* SNP genotypes dark-brown eye (X), hazel eye (Y) and blue eye (Z) color groups

	Eye Color Groups	Gene SNPs *rs12913832 - rs1800407* D'
X	Dark-Brown eye color	0.69
Y	Hazel eye color	0.98
Z	Blue eye color	0.80

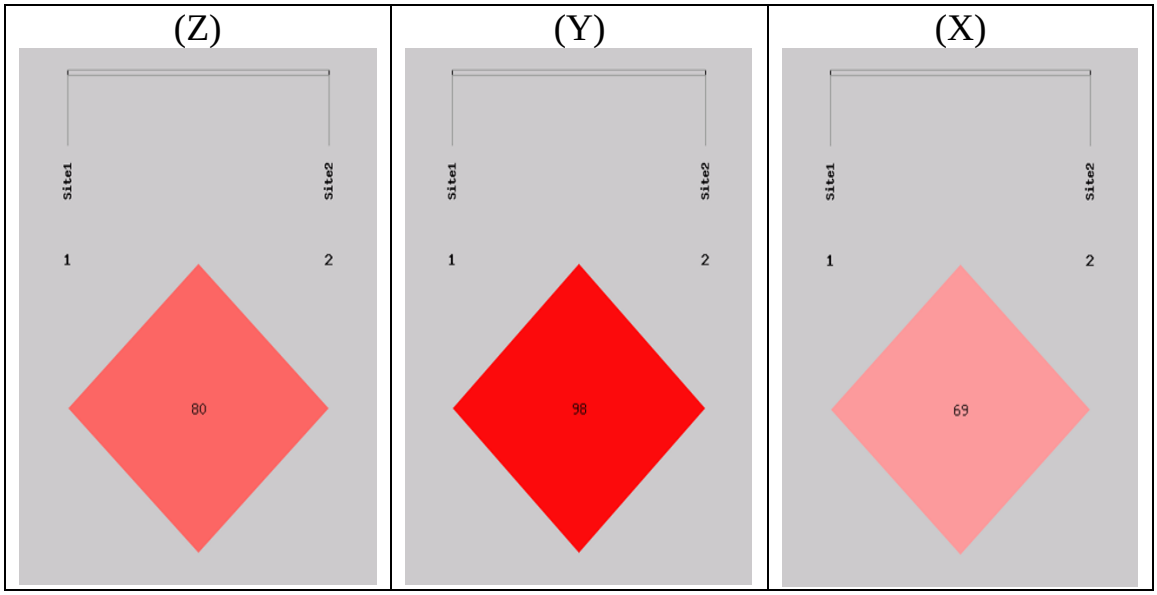

Figure 3. Linkage disequilibrium coefficient between two SNPs of dark-brown eye (X), hazel eye (Y) and blue eye (Z) color groups

Di-locus of chromosome 15 haplotype estimation between allele of SNPs (*rs12913832 - rs1800407*). The haplotype A-A manifested an increased frequency in the hazel eye *vs*. the dark-brown eye (0.349 – 0.143), the difference was significant (*p-v*=0.009), the OR=3.22; the haplotype A-G manifested an increased frequency in the hazel eye *vs*. the dark-brown (0.001- 0.157), the difference was significant (*p-v*=0.002). The OR=0.01;the haplotype G-A manifested a decreased frequency in the hazel eye vs. the dark-brown (0.501- 0.657), the difference was non-significant (*p-v*=0.083), the OR=0.52; the haplotype G-G manifested a decreased frequency in the hazel eye *vs*. the dark-brown

(0.149- 0.043), the difference was significant (*p-v*=0.048). The OR=3.94; which suggest the susceptibility role of such haplotype in increasing the recessive hazel eye color, (Table 6).

Table 6. Estimated haplotype frequencies between the SNPs *rs*1800407 and *rs*12913832 in hazel eye and dark-brown color subjects

Haplotype *rs1800407 – rs12913832*	N. (Frequency)		P- Value	OR (95% Cl)
	Hazel eye color	Dark-Brown eye color		
A-A	20.94(0.349)	8.55(0.143)	0.009	3.22 (1.31~7.91)
A-G	0.06(0.001)	9.45(0.157)	0.002	0.01 (0.00~0.08)
G-A	30.06(0.501)	39.45(0.657)	0.083	0.52 (0.25~1.09)
G-G	8.94(0.149)	2.55(0.043)	0.048	3.94 (0.93~16.62)

Di-locus of chromosome 15 haplotype estimation between allele of SNPs (*rs*12913832 - *rs*1800407). The haplotype A-A manifested an increased frequency in the blue eye *vs*. the dark-brown eye (0.042 – 0.143), the difference was significant (*p-v*=0.057), the OR=0.27; the haplotype A-G manifested a decreased frequency in the blue eye *vs*. the dark-brown (0.375- 0.157), the difference was significant (*p-v*=0.007), the OR=3.21; the haplotype G-A manifested a decreased frequency in the hazel eye *vs.* the dark-brown (0.475- 0.657), the difference was significant (*p-v*=0.043), the OR=0.47; the haplotype G-G manifested a decreased frequency in the hazel eye vs. the dark-brown (0.109- 0.043), the difference was non-significant (*p-v*=0.170), the OR=2.75; suggest the susceptibility role of such haplotype in increasing the recessive blue eye color (Table 7).

Table 7. Estimated haplotype frequencies between the SNPs *rs1800407* and *rs12913832* in blue eye and dark-brown color subjects

Haplotype *rs1800407 – rs12913832*	N. (Frequency)		P- Value	OR (95% Cl)
	Blue eye color	Dark-Brown eye color		
A-A	2.53(0.042)	8.55(0.143)	0.057	0.27 (0.06~1.13)
A-G	22.47(0.375)	9.45(0.157)	0.007	3.21 (1.34~7.65)
G-A	28.47(0.475)	39.45(0.657)	0.043	0.47 (0.23~0.98)
G-G	6.53(0.109)	2.55(0.043)	0.170	2.75 (0.62~12.23)

In previous study, sturm *et al*., (15) observed the regulatory region regulating constitutive expression of OCA2 was the conserved region around *rs12913832* and the G allele of *rs12913832* contributes to the reduction of OCA2 expression, especially within the pigment cells of the iris, which we assume is the ultimate cause of blue eye color, and the *rs12913832* polymorphic was directly associated with blue eye color in Iranian population (12), LD haplotype containing these SNPs were also significantly associated with the three categories eye colors dark-brown/hazel and blue iris pigmentation. These deviations provide an indication of the association with Shapturenko *et al*, (13). A study in Saudi population (1) observed the SNP *rs1800407* was not polymorphic in the Saudi population with the presence of the allele G. In this study, concluded the *rs1800407* indicating of eye color with the *rs12913832* because of the linkage disequilibrium coefficient between alleles of these SNPs strong associated with eye color. Irises exhibit extensive variation across Europe and a small degree of pigmentation from blue to green and brown in North Africa, Middle East, and Asia (8).

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