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Short Communication

Haplotypes from the *SLC45A2* gene are associated with the presence of freckles and eye, hair and skin pigmentation in Brazil

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ABSTRACT

The Solute Carrier Family 45, Member 2 (*SLC45A2*) gene encodes the Membrane-Associated Transporter Protein (MATP), which mediates melanin synthesis by tyrosinase trafficking and proton transportation to melanosomes. At least two *SLC45A2* coding SNPs [E272K (rs26722) and L374F (rs16891982)] were reported influencing normal variation of human pigmentation. Here we aimed at evaluating the influence of haplotypes of 12 SNPs within *SLC45A2* in the determination of eye, hair and skin pigmentation in a highly admixed population sample and comparing their frequencies with the ones found in data retrieved from the 1000 Genomes Project. To achieve this goal, 12 *SLC45A2* SNPs were evaluated in 288 unrelated individuals from the Ribeirão Preto city area, Southeastern Brazil. SNPs were genotyped by PCR-RFLP or Allele-specific PCR, followed by polyacrylamide gel electrophoresis. Haplotypes of each individual were inferred by two independent computational methods, PHASE and Partition-Ligation–Expectation-Maximization (PL-EM) algorithms, and 34 different haplotypes were identified. The hp9 haplotype was the most frequent (58.3%) and was associated with the presence of blond/red hair, pale skin, blue eyes and freckles. All haplotypes significantly associated with dark or light pigmentation features harbor the 374L and 374F alleles, respectively. These results emphasize the role played by haplotypes at *SLC45A2* in the determination of pigmentation aspects of human populations and reinforce the relevance of SNP L374F in human pigmentation.

1. Introduction

The *SLC45A2* (Solute Carrier Family 45, Member 2) gene, located at 5p13.2, is composed of seven exons that encode the Membrane-Associated Transporter Protein (MATP). MATP presents 12 transmembrane domains and mediates melanin synthesis by tyrosinase trafficking and proton transportation to melanosomes, and by controlling pH and ionic homeostasis within melanosomes [1–4].

Tyrosinase is an enzyme responsible for the conversion of tyrosine into L-DOPA, in the first step of the biochemical pathway towards melanin production. Melanosomal pH is a well-known factor that is crucial for melanin synthesis in two aspects: in early stage melanosomes, the acidic pH contributes to stabilize L-DOPA by preventing auto-oxidation and the increase of pH throughout melanosome maturation optimizes the tyrosinase activity for melanin production [5].

Under normal conditions, MATP elevates the melanosomal pH by functioning as a transporter using a proton gradient. Thus, copper can bind to tyrosinase, resulting in active tyrosinase. When mutated, the MATP protein is responsible for the development of oculocutaneous albinism type 4 (OCA4) [2,6]. In melanosomes from OCA4 affected subjects, MATP does not work properly and

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the melanosomal lumen becomes acidic. Hence, copper cannot be incorporated into tyrosinase, reducing its activity [5].

Former diversity studies regarding the *SLC45A2* coding region uncovered the existence of both synonymous and non-synonymous SNPs [4,7–9]. A guanine to adenine transition at exon 3 (refSNP ID: rs26722) and a guanine to cytosine transversion at exon 5 (refSNP ID: rs16891982) result in aminoacid exchanges at codons 272 (Glu272Lys or E272K) and 374 (Leu374Phe or L374F), respectively. These two coding SNPs (E272K and L374F) along with others on the *SLC45A2* gene have been studied in order to explain their relationships with normal variation in eye, hair and skin pigmentation, as well as to establish ancestry inferences [7,10–15]. Thus, in order to fully understand the *SLC45A2* contribution to the determination of human pigmentation, the nucleotide diversity of both promoter and coding region of this large gene, as well as the linkage disequilibrium (LD) patterns, are being studied [4,15–20]. In addition to pigmentation, some *SLC45A2* SNPs are also associated with diseases. For instance, rs35414 was found to be significantly associated with dark skin, dark hair and melanoma [21]. On the other hand rs28777 and rs35391, which are associated with pale skin, blue eyes and light hair, also provided some protection against this disease [22].

The analysis of sequence variability of a 7.55 kb region around the L374F position revealed evidence of recent positive selection favoring a haplotype harboring the 374F allele in Europe [18]. Considering L374F allele frequency estimates from ancient DNA deriving of prehistoric Europeans and modern Eastern Europeans, neutrality was overwhelmingly rejected and provided direct evidence that strong selection favoring lighter skin, hair and eye pigmentation has been operating in European populations over the last 5000 years [13]. Moreover, the analysis of African, European and Asian populations leaded to the identification of about 50 SNPs spread across *SLC45A2* exons and introns [4,18,23,24]. Yuasa et al. (2006) studied a set of 12 SNPs spanning more than 38 kb within *SLC45A2* and observed different haplotype distributions in Germans, Japaneses and Sub-Saharan Africans. In spite of the small sample sizes, 84.4% of the 32 haplotypes were found in single populations. It is likely that many of these haplotypes may be found at the Brazilian population, which is the result of intensive inter-ethnic crossings.

Notwithstanding the *SLC45A2* diversity and its potential informativeness, the correlation of these haplotypes with human pigmentation traits was not evaluated. Since the determination of eye, hair and skin pigmentation of unknown samples found in crime scenes would be of great value for forensic caseworks, the present study aimed at evaluating the influence of *SLC45A2* haplotypes in the determination of such pigmentation traits in a highly admixed population sample and comparing their frequencies with the ones found in the 1000 Genomes Project (http://www.1000genomes.org/) [25] dataset.

2. Material and methods

2.1. Laboratorial analysis

This study was approved in its ethical aspects by the "Comitê de Ética em Pesquisa" of this institution (Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, FFCLRP-USP) according to process CEP-FFCLRP n° 433/2008.

Blood samples were collected from 288 unrelated healthy individuals from the Ribeirão Preto city area, located at the Northern region of the State of São Paulo, Southeastern Brazil. Out of the 288 sampled individuals, 150 were randomly chosen and 138 were selected aiming at increasing less represented phenotypes. The total sample ($n = 288$) was used only in the association analyses, while the partial sample composed of randomly chosen individuals ($n = 150$) was used only in the remaining population genetics analyses. Individuals were classified according to their eye [blue ($n = 25$), green ($n = 75$), hazel ($n = 13$), light brown ($n = 68$), dark brown ($n = 107$)], hair [red ($n = 7$), blond ($n = 38$), brown ($n = 147$), and black ($n = 96$)], and skin [light/I + II ($n = 153$), intermediate/III + IV ($n = 94$), and dark/V + VI ($n = 41$)] pigmentation, and to the presence ($n = 46$) or absence ($n = 242$) of freckles. Skin pigmentation, ranging from I to VI, was defined according to the Fitzpatrick classification [26].

DNA was extracted from 10 mL of whole blood following a salting-out procedure [27]. A set of 12 SNPs were genotyped by PCR-RFLP or Allele-specific PCR according to conditions described previously [4,9]. The selected SNPs were: rs732740 (intron 1), rs250413 (intron 1), rs181832 (intron 2), rs3776549 (intron 2), rs3756462 (intron 2), rs26722 (exon 3 – E272K), rs2287949 (exon 4 – T329T), rs250417 (intron 4), rs16891982 (exon 5 – L374F), rs40132 (intron 5), rs35394 (intron 5), and rs3733808 (exon 7 – V507L). Amplicons were analyzed by 10% non-denaturing polyacrylamide gel electrophoresis followed by silver staining [28].

2.2. 1000 Genomes Project data

The 1000 Genomes Project Consortium [29] ran between 2008 and 2015, creating the largest public catalogue of human variation and genotype data. The project was conducted in four stages: a pilot phase and three phases of the main project (http://www.1000genomes.org/) [25]. In the final phase of the project, they reconstructed the genomes of 2504 individuals from 26 populations using a combination of low-coverage whole-genome sequencing, deep exome sequencing and dense microarray genotyping, and characterized a broad spectrum of genetic variation, in total over 88 million variants (SNPs, Indels, and structural variants), all phased onto high-quality haplotypes [29]. These 26 populations were divided into 5 super populations: African (AFR), Admixed American (AMR), East Asian (EAS), European (EUR), and South Asian (SAS). African was composed of: Esan in Nigeria; Gambian in Western Divisions in the Gambia; Luhya in Webuye, Kenya; Mende in Sierra Leone; and Yoruba in Ibadan, Nigeria. Admixed American was composed of: Americans of African Ancestry in southwestern USA; African Caribbeans in Barbados; Colombians from Medellin, Colombia; Mexican Ancestry from Los Angeles, USA; Peruvians from Lima, Peru; and Puerto Ricans from Puerto Rico. East Asian was composed of: Chinese Dai in Xishuangbanna, China; Han Chinese in Bejing, China; Southern Han Chinese; Japanese in Tokyo, Japan; and Kinh in Ho Chi Minh City, Vietnam. European was composed of: Utah Residents with Northern and Western European Ancestry; Finnish in Finland; British in England and Scotland; Iberian Population in Spain; and Toscani in Italia. South Asian was composed of: Bengali from Bangladesh; Gujarati Indian from Houston, Texas; Indian Telugu from the UK; Punjabi from Lahore, Pakistan; and Sri Lankan Tamil from the UK.

The 1000 Genomes Project maintains a specific version of the Ensembl Browser, based on GRCh37, to visualize its variants. This browser (Phase 3), hosted at http://browser.1000genomes.org [30], was used to download the VCF file with phased variation sites on the *SLC45A2* gene. Only the 12 SNPs that were genotyped for the Brazilian population in the present study was retrieved from 1000 Genomes Project VCF file.

2.3. Statistical analysis

Given that some samples may present two or more heterozygous SNPs, resulting in more than one possible haplotype combination, two independent computational methods were used to determine the haplotypes of each subject, without taking into account any prior information: (1) the Expectation-Maximization

(EM) algorithm [31] implemented at the PL-EM software [32] and (2) a coalescence based method implemented at the PHASE v2 software [33]. Only the individuals that presented the same haplotype pairs estimated by both methods were included in the haplotype analyses. The LD pattern was evaluated by calculating D' and log of odds (LOD) scores, and LD plots were produced using Haploview 4.2 [34], considering only variable sites with a minimum allele frequency (MAF) of 1%.

Allele, genotype, haplotype and protein frequencies, as well as the observed heterozygosity (h_O), were computed by the direct counting method. Adherence of genotypic proportions to expectations under Hardy-Weinberg equilibrium was verified by the exact test of Guo and Thompson [35], by means of the ARLEQUIN version 3.5.1.2 program [36]. The ARLEQUIN software was also used to estimate expected heterozygosity values (h_{Sk}) and haplotype diversities, and to perform the pairwise exact test of sample differentiation based on haplotype frequencies [37], when the whole sample was stratified according to eye, hair and skin pigmentation phenotypes. Additionally, haplotype and protein frequencies were compared between groups using the Fisher exact test implemented in the GraphPad InStat 3.06 software. Odds Ratio and its 95% Confidence Interval was used to estimate the magnitude of significant associations. Since within each group 34 haplotypes and 4 proteins were used for comparisons, the conservative Bonferroni correction was used to adjust the significance level for multiple testing, resulting in $\alpha = 0.0015$ (i.e., 0.05/34) for haplotypes and $\alpha = 0.0125$ (i.e., 0.05/4) for proteins.

3. Results

All the 288 individuals were successfully genotyped for the 12 SNPs, which presented genotype distributions according to Hardy-Weinberg Equilibrium expectations, except for SNP rs16891982 (L374F) that showed heterozygote deficiency. Of the total sample ($n = 288$ individuals) submitted to computational reconstruction by PHASE and PL-EM methodologies, 57 and 31 had their most probable haplotype constitutions reconstructed with a probability of less than 80% by the respective methods. Both methods presented the same haplotype constitutions in 270 (93.75%) of the 288 individuals, with average probabilities of 0.9339 and 0.9620, respectively. Based on a conservative attitude, it was considered for the subsequent analyses only those 270 individuals (or 139 individuals in the case of the partial sample composed of randomly chosen individuals) whose haplotype constitution was found to be the same by the two methods. LD patterns observed in the Brazilian sample reveal the existence of two haplotype blocks (Fig. 1), presenting a similar pattern previously observed in the German and Japanese population samples [4]. A total of 34 different (8 private) haplotypes were identified for the total sample while only 23 different (4 private) haplotypes were identified for the partial sample (Table 1).

Haplotype hp9 was the most frequent in the Brazilian sample (62.95% in the partial sample), as well as in the EUR (89.20%) and AMR (34.00%) samples from the 1000 Genomes Project, although much less frequent in AFR (0.50%), SAS (5.32%) and EAS (0.40%). Haplotype hp1 was the second most frequent in our sample (7.55%), AFR (24.60%) and SAS (28.40%), but was the most frequent in EAS (26.70%) and low frequent in EUR (1.49%) and AMR (8.53%). The most frequent haplotypes in AFR (hp2 = 26.50%) and SAS (hp15 = 36.40%) were observed in Brazil in frequencies around 5%. The observed haplotype diversity in the Brazilian population (0.5900) is higher than in EUR (0.2040), but lower than those of AFR (0.8313), EAS (0.8560) and SAS (0.7600) (Table 1).

The rs16891982 (L374F) allele frequencies in our partial sample was different from those found in the populations from the Yuasa

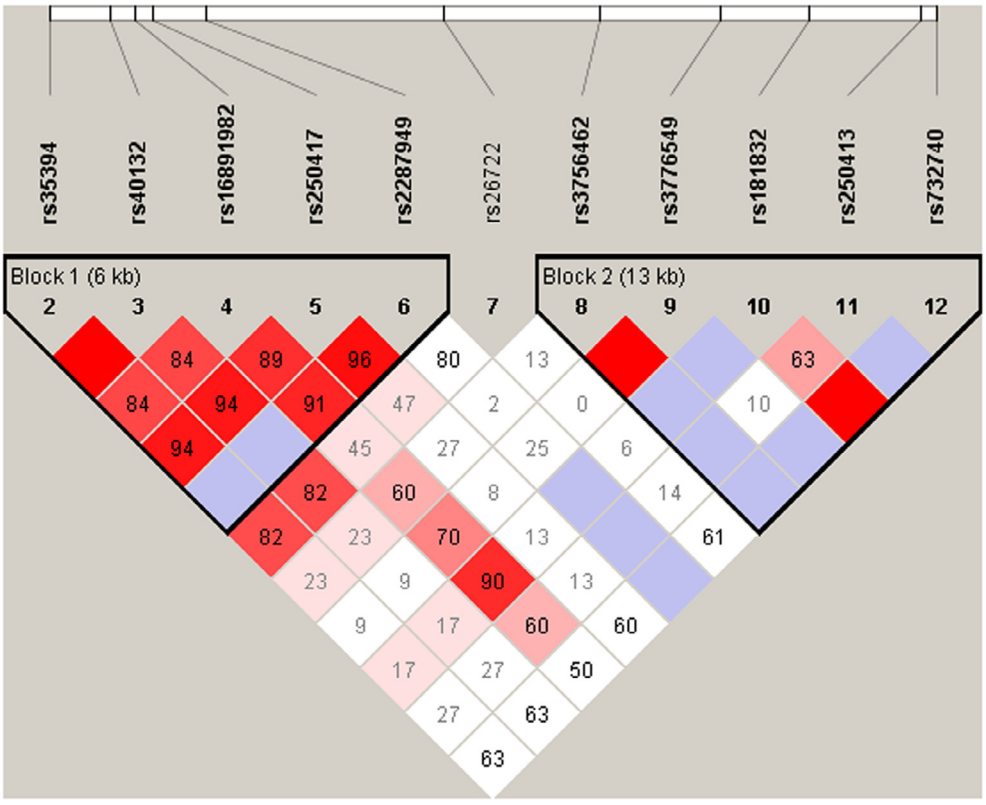

Fig. 1. Linkage Disequilibrium between pairs of variable sites at the *SLC45A2* coding region. Areas in red (or dark gray) indicate strong LD (LOD $\geqslant 2$, $D' = 1$); areas in light blue (or light gray) indicate moderate LD (LOD $\geqslant 2$, $D' = 1$); white boxes indicate weak LD (LOD < 2, $D' < 1$). D' values different from 1.00 are represented inside the squares as percentages. The haplotype blocks were defined according to the Solid Spine of LD method implemented in Haploview 4.2 [34]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
SLC45A2 haplotype frequencies and diversity in a Brazilian population sample, compared with data from five populations studied by the 1000 Genomes Project and from African, Japanese and German samples [4]. Haplotypes were determined by the following SNPs: rs732740, rs250413, rs181832, rs3776549, rs3756462, rs26722 (E272K), rs2287949 (T329T), rs250417, rs16891982 (L374F), rs40132, rs35394, and rs3733808 (V507L).

Haplotypes		Brazil		Yuasa			The 1000 Genomes Project				
		Partial	Total	African	Japanese	German	AFR	AMR	EAS	EUR	SAS
hp1	TCTGTGGGGTAG	0.0755	0.0704	0.4490	0.2450		0.2460	0.0853	0.2670	0.0149	0.2840
hp2	TCCGTGGGGTAG	0.0504	0.0426	0.1160			0.2650	0.1000	0.0050	0.0030	0.0143
hp3	TCCATGGGGTAG			0.1090							
hp4	TCTATGGGGTAG	0.0252	0.0259	0.0850			0.1360	0.0377			0.0041
hp5	TCCGTAGGGTAG			0.0340			0.0020				
hp6	TTCGTGGGGTAG	0.0180	0.0111		0.0560		0.0536	0.0397	0.0556	0.0010	0.0082
hp7	TCTACGGGGTAG	0.0216	0.0167		0.0530	0.0050	0.0020	0.0060	0.0536	0.0050	0.0041
hp8	TCTGTGAGGTAG				0.0050						
hp9	TCTGTGGGCTAG	0.6295	0.5830			0.8960	0.0050	0.3400	0.0040	0.8920	0.0532
hp10	TCTACGGGCTAG	0.0072	0.0093			0.0390		0.0010	0.0020	0.0189	0.0020
hp11	TTCGTGGGCTAG					0.0110		0.0020		0.0109	
hp12	TCCGTGGGCTAG	0.0036	0.0037			0.0050		0.0030		0.0070	0.0031
hp13	CCCGTGGGCTAG		0.0019			0.0050		0.0030		0.0010	
hp14	TCTGTAGGCTAG	0.0036	0.0463			0.0050				0.0010	
hp15	TCTGTGACGTAG	0.0647	0.0574	0.0480	0.1020	0.0050	0.1190	0.0685	0.0774	0.0099	0.3640
hp16	TCTATGACGTAG			0.0310			0.0020				0.0020
hp17	TCCATGACGTAG			0.0100							
hp18	TCTACGACGTAG		0.0037		0.0590		0.0040	0.0060	0.0427	0.0010	0.0072
hp19	TTCGTGACGTAG				0.0550		0.0010	0.0090	0.0546		0.0184
hp20	TCTGTGACGTAC				0.0050						0.0020
hp21	TTCACGACGTAG				0.0030						
hp22	TTCGTAACGTAG				0.0100						
hp23	TCTGTGGCGTAG			0.0640			0.0188	0.0010			0.0010
hp24	TCCGTAGCGTAG			0.0250			0.0030				
hp25	TCTGTAGCGCGG	0.0396	0.0389		0.2560	0.0230	0.0040	0.1720	0.1910	0.0129	0.1490
hp26	TCTACAGCGCGG		0.0111		0.0780	0.0040	0.0010	0.0070	0.0843	0.0070	0.0051
hp27	TTCGTAGCGCGG	0.0108	0.0056		0.0390			0.0347	0.1080		0.0123
hp28	TCCATGGCGCGG			0.0290							
hp29	TCTGTGGCGCGG	0.0072	0.0056		0.0140		0.0119	0.0080	0.0367	0.0010	0.0020
hp30	TCTACGGCGCGG				0.0130				0.0040		
hp31	TCTGTGGCGCAG				0.0050						
hp32	TTCACGGCGCGG				0.0030						
hp33	TTTGTGGGCTAG	0.0036	0.0056					0.0010			
hp34	TCCGTGGCGTAG		0.0037				0.0337	0.0149			0.0010
hp35	TTCGTGGGGCGG	0.0036	0.0019								
hp36	TCTGTAGCCCGG	0.0036	0.0037								
hp37	TCCGTAGCGCGG	0.0108	0.0074				0.0020	0.0030		0.0010	0.0020
hp38	TCCGTGGCGCGG	0.0036	0.0019				0.0129	0.0080			
hp39	TCTGTGAGCTAG	0.0036	0.0019								
hp40	TCTACGGCCCGG		0.0019								
hp41	TCTGTGGCCCGG		0.0019								
hp42	TCTGTAGCGTAG	0.0036	0.0056				0.0208	0.0080			
hp43	TCTATGGGCTAG	0.0036	0.0037					0.0020		0.0070	
hp44	CCCGTAGCGCGG	0.0036	0.0037							0.0010	
hp45	TCTGTGACCTAG	0.0036	0.0019								
hp46	TTTATGGGGTAG		0.0019								
hp47	TCTGTAGGGTAG		0.0148				0.0060				0.0020
hp48	TCTGTAGGGCGG		0.0019						0.0010		
hp49	TTTGTAGCCCGG		0.0019								
hp50	TCTGTAACGTAG		0.0019					0.0020	0.0010		
hp51	TCTGCGGGCTAG									0.0010	
hp52	TTTGTAGCGCGG								0.0010	0.0010	0.0020

Table 1 (*continued*)

Haplotypes		Brazil		Yuasa		The 1000 Genomes Project						
		Partial	Total	African	Japanese	German	AFR	AMR	EAS	EUR	SAS	
hp53	TTCGTGGCGCGG							0.0050	0.0040			
hp54	TTCGTAGGGTAG								0.0010			
hp55	TCTACAGGGTAG								0.0010			
hp56	TCTATGGCGCGG						0.0268	0.0050			0.0184	
hp57	TCCGTGACGTAG						0.0149	0.0109	0.0010			
hp58	TCTACAGGCTAG							0.0010				
hp59	TCTATACGCGCG							0.0020			0.0327	
hp60	TCTGCGACGTAG							0.0010			0.0010	
hp61	TCTGTGGGCAG						0.0010	0.0020				
hp62	CCCGTGGCGTAG									0.0020		
hp63	TTTGTGGGTAG								0.0020	0.0010	0.0010	
hp64	TCTATAGCGTAG						0.0020	0.0010				
hp65	TCCGCGGCCCGG								0.0010			
hp66	TCCGCGGGGTAG							0.0010				
hp67	TCTATAACGTAG										0.0010	
hp68	TTCGTGGCCTAG						0.0030					
hp69	TTTGTGACGTAG										0.0020	
hp70	TCTGTAGCGCAG								0.0010			
hp71	TCCGTGGGGCAG						0.0010					
hp72	TCTATGGCGTAG						0.0010					
hp73	TCTATCGGGTGG						0.0010					
	Haplotype diversity	0.5900 ± 0.0341	0.6454 ± 0.0230	0.8150 ± 0.0510	0.8810 ± 0.0170	0.2610 ± 0.0430	0.8313 ± 0.0061	0.8284 ± 0.0082	0.8560 ± 0.0056	0.2040 ± 0.0173	0.7600 ± 0.0083	
	n[a]	139	270	17	103	92	504	504	504	502	489	

[a] Number of sampled individuals.

et al. (2006) study and some populations from the 1000 Genomes Project. The 374F (rs16891982*C) allele showed a 96.20% frequency in German and was absent in Japanese and African [4]. A similar pattern is observed in the 1000 Genomes Project samples, where the 374F allele registered a 93.84% frequency for the EUR sample, very low frequencies in AFR (0.0050), EAS (0.0059) and SAS (0.0583) samples, and an intermediate frequency (0.3621) for the AMR sample. In our partial sample, the 374F allele presented a 64.33% frequency (Table 2). The rs3733808, which is associated with OCA4 in a Japanese sample [38], was monomorphic, being absent in all samples except for Japanese (0.0049) and SAS (0.0020).

The intrapopulational genetic diversity estimated by the expected heterozygosity (H_S) was 0.1619 in Brazil. African (0.1940) and Japanese (0.2530) from the Yuasa et al. (2006) study and AMR (0.2445), EAS (0.2963) and SAS (0.2023) populations presented a higher diversity, while German (0.1100), AFR (0.1542), and EUR (0.0475) presented lower values. It is noteworthy that H_S was lower than 0.3 for all the SNPs in our study, except for rs16891982 (L374F) (0.4605), which was similar to the observed in the AMR (0.4624) sample from the 1000 Genomes Project (Supplementary Table S1).

For association analyses, the total sample ($n = 288$) was taken into account. The exact test of sample differentiation based on haplotype frequencies ($n = 270$, after the exclusion of the 18 individuals for which both methods for haplotype inference presented different haplotype constitutions) revealed significantly different haplotype distributions in all three comparisons regarding skin pigmentation (Supplementary Table S2). The same pattern can be observed in most of the comparisons that involved hair (specially blond *vs.* black, brown *vs.* black, and red/blond *vs.* black) and eyes (specially green *vs.* dark brown, blue/green *vs.* light/dark brown, blue/green *vs.* dark brown) (Supplementary Table S2). Haplotype hp9, for instance, was the most frequent (58.30%, Table 1) and was associated with light phenotypes, such as the presence of blond/red hair, pale skin, blue eyes and freckles (Table 3 and Supplementary Tables S3, S4 and S5). On the other hand, hp1 and hp25 haplotypes were associated with dark phenotypes, with black hair (mainly hp25), dark-brown eyes and dark skin (mainly hp1) (Table 3 and Supplementary Tables S3, S4 and S5).

Table 4 shows the significantly protein associations with *p*-values lower than 0.05. Protein EF (61.48%) was highly associated with lighter pigmentation features: blond hair ($p = 4.06 \times 10^{-7}$; OR = 5.1784), blue eyes ($p = 0.0009$; OR = 3.4247), light skin ($p = 5.87 \times 10^{-12}$; OR = 3.5172) and presence of freckles ($p = 0.0001$; OR = 2.9774). On the other hand, protein EL (24.26%) was significantly associated with darker pigmentation: black hair ($p = 9.17 \times 10^{-12}$; OR = 4.1937), dark brown eyes ($p = 6.01 \times 10^{-9}$; OR = 3.3279), dark skin ($p = 2.94 \times 10^{-12}$; OR = 5.9689) and absence of freckles ($p = 0.0001$; OR = 3.9778). Proteins KL (9.07%) and KF (5.19%) were also associated with darker and lighter features, respectively.

4. Discussion

The study of the genetics of human pigmentation is of paramount importance for understanding human biology and evolution, as well as skin cancer biology, and allows the selection of DNA variation sites that act as predictors of eye, hair and skin color for use in practical applications such as in forensics [39]. In this context, *SLC45A2* gene and its polymorphisms have been studied in the attempt to explain the normal variation of human pigmentation along with estimating population and individual ancestries. Many articles have shown significant differences in the allele frequencies on *SLC45A2* polymorphisms within and between different

Table 2
SLC45A2 allele frequencies of 12 SNPs in a Brazilian population sample, compared with data from five populations studied by the 1000 Genomes Project and from African, Japanese and German samples [4].

SLC45A2	Position at Chr5[a]	dbSNP ID	Region	Mutation[b]	Brazil	Yuasa et al., 2006			The 1000 Genomes Project				
						African	Japanese	German	AFR	AMR	EAS	EUR	SAS
1	33983189	rs732740	Intron 1	T > C	0.0033	0.0000	0.0000	0.0054	0.0000	0.0030	0.0000	0.0039	0.0000
2	33982568	rs250413	Intron 1	C > T	0.0400	0.0000	0.1650	0.0109	0.0575	0.0913	0.2262	0.0139	0.0439
3	33978188	rs181832	Intron 2	T > C	0.1100	0.3235	0.1650	0.0217	0.3919	0.2341	0.2301	0.0268	0.0777
4	33974742	rs3776549	Intron 2	G > A	0.0800	0.2647	0.2087	0.0489	0.1756	0.0774	0.1875	0.0387	0.0583
5	33970006	rs3756462	Intron 2	T > C	0.0467	0.0000	0.2087	0.0489	0.0069	0.0318	0.1885	0.0328	0.0194
6	33963870	rs26722 (E272K)	Exon 3	G > A (E > K)	0.0900	0.0588	0.3835	0.0326	0.0407	0.2302	0.3898	0.0238	0.2065
7	33954511	rs2287949 (T329T)	Exon 4	G > A	0.0800	0.0882	0.2379	0.0054	0.1409	0.0972	0.1766	0.0109	0.4161
8	33952378	rs250417	Intron 4	G > C	0.1767	0.2059	0.6408	0.0326	0.2818	0.3661	0.6081	0.0348	0.6237
9	33951693	rs16891982 (L374F)	Exon 5	G > C (L > F)	0.6433	0.0000	0.0000	0.9620	0.0050	0.3621	0.0059	0.9384	0.0583
10	33950703	rs40132	Intron 5	T > C	0.1000	0.0294	0.4078	0.0272	0.0605	0.2460	0.4325	0.0238	0.2055
11	33948319	rs35394	Intron 5	A > G	0.1000	0.0294	0.4029	0.0272	0.0595	0.2441	0.4315	0.0238	0.2055
12	33944827	rs3733808 (V507L)	Exon 7	G > C (V > L)	0.0000	0.0000	0.0049	0.0000	0.0000	0.0000	0.0000	0.0000	0.0020
				n[c]	150	17	103	92	504	504	504	502	489

[a] SNP position retrieved from The 1000 Genomes Browser/GRCh37 assembly.
[b] Frequencies of the derived allele are presented.
[c] Number of sampled individuals.

Table 3
SLC45A2 haplotypes associated with different condition of a given trait (eye, hair and skin pigmentation and freckles) in a Brazilian population sample. Only the significant associations (p-value $\leqslant 0.05$) and OR values higher than 1.0 are reported.

Trait	Group	*n*[b]	Haplotype[c]	Haplotype frequency in the given group	*p*-Value[d]	OR (95%C.I.)	
Eyes	Blue	24	hp9	0.8120	**0.0006**	3.3913	(1.6078–7.1532)
	Blue + green	94	hp9	0.6809	**0.0009**	1.8824	(1.2985–2.7287)
	Blue + green	94	hp14	0.0957	**0.0001**	5.2185	(2.1384–12.7350)
	Green	70	hp33	0.0214	0.0171	20.3891	(1.0465–397.2290)
	Green	70	hp14	0.1000	**0.0015**	3.9293	(1.7395–8.8756)
	Hazel	13	hp41	0.0385	0.0481	60.5294	(2.4059–1522.8561)
	Hazel	13	hp26	0.0769	0.0297	10.625	(1.8537–60.9016)
	Hazel	13	hp42	0.0769	0.0065	42.7500	(3.7444–488.0776)
	Hazel	13	hp14	0.1540	0.0268	4.2684	(1.3497–13.4992)
	Light brown	65	hp9	0.6690	0.0248	1.6151	(1.0675–2.4435)
	Light + dark brown	163	hp1	0.0951	0.0055	3.1075	(1.3426–7.1925)
	Light + dark brown	163	hp2	0.0613	0.0079	4.5969	(1.3489–15.6660)
	Light + dark brown	163	hp15	0.0767	0.0219	2.8793	(1.1609–7.1414)
	Dark brown	98	hp1	0.1220	**0.0007**	3.2890	(1.6589–6.5209)
	Dark brown	98	hp2	0.0816	**0.0013**	4.2794	(1.7287–10.5934)
	Dark brown	98	hp15	0.0969	0.0038	2.9699	(1.4094–6.2582)
	Dark brown	98	hp25	0.0663	0.0189	2.9836	(1.2143–7.3308)
Hair	Red	7	hp49	0.0714	0.0259	117.0000	(4.5553–3005.0643)
	Red + blond	43	hp9	0.7791	$\mathbf{4.42 \times 10^{-5}}$	2.9291	(1.7037–5.0359)
	Blond	36	hp9	0.8060	$\mathbf{3.08 \times 10^{-5}}$	3.4013	(1.8456–6.2685)
	Blond	36	hp33	0.0278	0.0481	13.3429	(1.1941–149.0893)
	Brown	140	hp9	0.6680	$\mathbf{3.97 \times 10^{-5}}$	2.0736	(1.4645–2.9361)
	Black	87	hp1	0.1440	$\mathbf{1.51 \times 10^{-5}}$	4.5560	(2.2692–9.1473)
	Black	87	hp2	0.0747	0.0201	2.8745	(1.2346–6.6929)
	Black	87	hp15	0.1150	**0.0002**	4.1913	(1.9608–8.9589)
	Black	87	hp25	0.0747	0.0069	3.6134	(1.4689–8.8887)
	Black	87	hp26	0.0287	0.0148	10.7988	(1.2519–93.1490)
Skin[a]	Light (I + II)	144	hp9	0.7150	$\mathbf{3.83 \times 10^{-11}}$	3.2958	(2.3058–4.7108)
	Light (I + II)	144	hp14	0.0799	$\mathbf{4.03 \times 10^{-5}}$	10.8491	(2.5317–46.4920)
	Dark (V + VI)	40	hp1	0.2250	$\mathbf{5.92 \times 10^{-7}}$	6.3871	(3.2034–12.7348)
	Dark (V + VI)	40	hp2	0.1130	0.0031	4.0382	(1.6850–9.6779)
	Dark (V + VI)	40	hp4	0.0625	0.0424	3.3407	(1.0898–10.2410)
	Dark (V + VI)	40	hp15	0.1130	0.0336	2.5237	(1.1170–5.7018)
	Dark (V + VI)	40	hp25	0.0875	0.0245	3.0548	(1.1928–7.8236)
	Dark (V + VI)	40	hp47	0.0500	0.0196	6.0000	(1.4692–24.5026)
Freckles	Presence	41	hp9	0.7930	2.8×10^{-5}	3.1812	(1.8086–5.5955)

[a] Skin color types, ranging from I to VI, were defined according to the Fitzpatrick classification [26].
[b] Number of individuals within a given group.
[c] Haplotypes defined at Table 1.
[d] Probability values obtained by means of the two-sided Fisher exact test when comparing a given group with a group composed by the remaining samples. Statistically significant values at a 5% significance level after Bonferroni correction are highlighted in boldface ($p < 0.0015$).

Table 4
SLC45A2 proteins associated with different condition of a given trait (eye, hair and skin pigmentation and freckles) in a Brazilian population sample. Only the significant associations (p-value $\leqslant 0.05$) and OR values higher than 1.0 were reported.

SNPs		Protein[a]	Feature	p-Value[f]	OR	(95%CI)
rs26722 (E272K)	rs16891982 (L374F)					
G (272E)	C (374F)	EF[b]	Blond hair	$\mathbf{4.06 \times 10^{-7}}$	5.1784	(2.5154–10.6608)
			Red + blond hair	$\mathbf{1.76 \times 10^{-6}}$	3.8374	(2.1019–7.0056)
			Brown hair	**0.0001**	2.0597	(1.4480–2.9298)
			Blue eyes	**0.0009**	3.4247	(1.5697–7.4717)
			Light brown eyes	0.0131	1.7149	(1.1201–2.6257)
			Blue + green eyes	**0.0006**	1.9300	(1.3203–2.8213)
			Light skin	$\mathbf{5.87 \times 10^{-12}}$	3.5172	(2.4441–5.0616)
			Presence of freckles	**0.0001**	2.9774	(1.6723–5.3011)
G (272E)	G (374L)	EL[c]	Black hair	$\mathbf{9.17 \times 10^{-12}}$	4.1937	(2.7733–6.3416)
			Brown + black hair	**0.0000**	6.2232	(2.4646–15.7135)
			Dark brown eyes	$\mathbf{6.01 \times 10^{-9}}$	3.3279	(2.2151–4.9998)
			Brown eyes	$\mathbf{6.65 \times 10^{-8}}$	3.4354	(2.1443–5.5040)
			Intermediate skin	**0.0097**	1.7295	(1.1496–2.6018)
			Dark skin	$\mathbf{2.94 \times 10^{-12}}$	5.9689	(3.6133–9.8600)
			Absence of freckles	**0.0001**	3.9778	(1.7846–8.8664)
A (272K)	G (374L)	KL[d]	Black hair	$\mathbf{1.69 \times 10^{-5}}$	3.8048	(2.0744–6.9788)
			Brown + black hair	0.0134	4.8501	(1.1555–20.3575)
			Dark brown eyes	**0.0027**	2.5635	(1.4129–4.6510)
			Dark skin	**0.0010**	3.2348	(1.6848–6.2109)
A (272K)	C (374F)	KF[e]	Green eyes	**0.0014**	3.5723	(1.6547–7.7121)
			Hazel eyes	0.0392	3.7121	(1.1855–11.6236)
			Blue + green eyes	**0.0003**	4.2847	(1.8981–9.6722)
			Light skin	**0.0001**	7.8897	(2.3526–26.4590)

[a] Proteins were labeled considering the primary sequence of the longest isoform (Q9UMX9-1) available at the UniProt database (which includes 272E and 374L amino acids (EL).
[b] Determined by the following haplotypes found in Brazil: hp9, hp10, hp12, hp13, hp33, hp39, hp40, hp41, hp43, hp45.
[c] Determined by the following haplotypes found in Brazil: hp1, hp2, hp4, hp6, hp7, hp15, hp18, hp29, hp34, hp35, hp38, hp46.
[d] Determined by the following haplotypes found in Brazil: hp25, hp26, hp27, hp37, hp42, hp44, hp47, hp48, hp50.
[e] Determined by the following haplotypes found in Brazil: hp14, hp36, hp49.
[f] Probability values obtained by means of the two-sided Fisher exact test when comparing a given group with a group composed by the remaining samples. Statistically significant values at a 5% significance level after Bonferroni correction are highlighted in boldface ($p < 0.0125$).

populational groups [4,7,17,24,40–43]. Moreover, many variation sites present significant associations with pigmentation phenotypes. For example, the 374F allele was associated with light pigmentation phenotypes (blond hair, blue eyes and light skin) in association studies considering populations of different ancestries [4,16,44,45].

In our partial sample, which was composed of randomly chosen individuals, the 374F (rs16891982*C) allele presented a 64.33% frequency, which is an intermediate frequency when compared to EUR (0.9384) sample and AFR (0.0050), EAS (0.0059) and SAS (0.0583) samples (Table 2). Furthermore, this intermediate frequency is similar to that of the AMR (0.3621) sample, clearly reflecting the admixed nature of the Brazilian population [46]. The intrapopulational genetic diversity estimated by the expected heterozygosity was lower than 0.3 for all the SNPs in the Brazilian sample (Supplementary Table S1), except for the rs16891982 (L374F) (0.4605). This was the only *locus* that showed deviation from Hardy-Weinberg equilibrium, which was due to heterozygotes deficiency. One possible explanation for this deviation is the occurrence of inter-ethnic admixture, particularly concerning Europeans and Africans, associated with population substructure. Our conclusion is corroborated by the 1000 Genomes dataset, since AMR was the only group revealing high expected heterozygosity (0.4624) and Hardy-Weinberg disequilibrium ($p = 0.0000$) due to heterozygotes deficiency.

In the Yuasa et al. (2006) study, the 272K allele showed a 38.35% frequency in the Japanese sample and was very low in African (0.0588) and German (0.0326) samples. The same pattern can be observed in the 1000 Genomes samples, in which the 272K allele registered a higher frequency for Asians (EAS – 0.3898 and SAS – 0.2065) and lower frequency for AFR (0.0407) and EUR (0.0238) samples. In addition, the 272K allele has also shown low frequencies in another set of Caucasians (0.028) and Australian Aborigines (0.029), and high frequencies in Asians (0.339), suggesting the 272K allele as an Asian ancestry informative marker [16]. This trend can be verified in another study that presents the 272K allele in higher frequency in an Asian population (0.8) than in African (0.05) and European (0.03) populations [18]. Given that Africans, Australian Aborigines and Caucasians presented similar frequencies despite having very different skin colors, one may conclude that this polymorphism, in spite of the amino acid substitution, is not related with the determination of skin pigmentation; it is rather reflecting demographic events.

The Brazilian intermediate haplotype diversity (0.5900) is also expected since the Brazilian population is characterized by the admixture of three founder populations (Africans, Europeans and Native Americans), with higher contribution of Europeans. It is noteworthy that haplotype diversity in EUR (0.2040) is significantly lower than in AFR, EAS and SAS (Table 1), which reflects the already mentioned positive selection favoring lighter skin, hair, and eye pigmentation in European populations over the last 5000 years [13]. It is also worth mentioning that 93.87% of EUR haplotypes harbor the 374F allele, which is systematically associated with light pigmentation, while only 1.92% of AFR haplotypes include such allele. The inter-ethnic admixture levels of Ribeirão Preto area has already been characterized by autosomal STRs. The gene identity method resulted in 79% European, 14% African, and 7% Amerindian contributions for white individuals [47], 62%, 26% and 12% of European, African and Amerindian contribution respectively, for mulattos and 37% and 63% of European and African contribution, respectively, for black individuals [48]. It should be mentioned that the present partial sample is composed mainly

by white and intermediate individuals (84%), suggesting a higher European ancestry.

Regarding the proteins analyses, protein EF exhibit an association with light pigmentation phenotypes. Haplotype 9 (hp9), one of those that originate such protein, showed association with all the features analyzed (hair, skin and eye color and freckles). The same haplotype was also more frequent and associated with European ancestry in the Yuasa et al. (2006) study. Two haplotypes (hp42 and hp47), which had not been observed by Yuasa et al., originate protein KL and were associated with hazel eyes and dark skin, respectively (Table 3). Both haplotypes are found in the 1000 Genomes dataset (Table 1): while hp42 is found exclusively in AFR (0.0208) and afro-derived samples from the AMR (0.0080) group, hp47 is found both in AFR (0.0060) and in darkly pigmented SAS (0.0020).

Ultimately, all haplotypes/proteins significantly associated with dark or light pigmentation features harbor the 374L and 374F alleles, respectively. It should be emphasized that the 374F allele is fixed or almost fixed in Caucasian populations, while it is almost absent in other worldwide populations examined so far (Table 2) [4,7,9,12,39,49]. In addition to the 374L allele, the 272K allele has been associated with dark eye, hair and skin pigmentation [3,15,16,24]. However, since the 374L and 272K alleles are in strong LD within Europe and since the 272K allele is rare in African populations, it was proposed that only the 374L allele played a major role on pigmentation of Europeans. Accordingly, it would be expected that individuals harboring the 374F allele would present pale skin and selective advantage regarding vitamin D synthesis in low latitude regions [18], irrespective of the E272K genotype. Protein associations, however, indicate that E272K modifies L374F associations. Considering the two pigmentation phenotypes (i.e., blue + green eyes and light skin) that resulted in significant associations for both proteins that include the 374F allele (EF and KF), the presence of the 272K allele strengthens the magnitude of the associations, as can be observed by the higher odds ratio. Similarly, considering the four pigmentation phenotypes (i.e., black hair, brown + black hair, dark brown eyes and dark skin) that resulted in significant associations for both proteins that include the 374L allele (EL and KL), the presence of the 272E allele strengthens the magnitude of the associations. Finally, other variation sites (Supplementary Tables S6 and S7) with different levels of LD with L374F also influence the analyzed pigmentation features. For instance, the SNPs rs35394, rs40132, rs250417, and rs2287949 compose the first haplotype block along with L374F, while SNPs rs3776549 and rs181832 are placed within the second haplotype block (Fig. 1). These findings suggest that other neighboring SNPs besides L374F add to SLC45A2 influence on eye, hair and skin pigmentation in Brazil.

5. Conclusion

The distribution of *SLC45A2* alleles and haplotypes in the current Brazilian population sample is consistent with its interethnic admixture history. A similar situation is observed in the AMR sample of the 1000 Genomes Project. The observed phenotype associations emphasize that *SLC45A2* haplotypes are engaged in the determination of human pigmentation, both reinforcing the relevance of SNP L374F in eye, hair and skin color, but also suggesting an important role played by its surrounding variation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.legalmed.2016.12.013.

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