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ORIGINAL INVESTIGATION

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The 8818G allele of the agouti signaling protein (*ASIP*) gene is ancestral and is associated with darker skin color in African Americans

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Abstract Skin color, a predictor of social interactions and risk factor for several types of cancer, is due to two contrasting forms of melanin, the darker eumelanin and lighter phaeomelanin. The lighter pigment phaeomelanin is the product of the antagonistic function of the agouti signaling protein (ASIP) on the α-melanocyte stimulating hormone receptor (MC1R). Studies have shown that a single-nucleotide polymorphism (SNP) in the 3′UTR of the *ASIP* gene is associated with dark hair and eyes; however, little is known about its role in inter-individual variation in skin color. Here we examine the relationship between the *ASIP* g.8818A > G SNP and skin color (M index) as assessed by reflectometry in 234 African Americans. Analyses of variance (ANOVA) were performed to evaluate the effects of *ASIP* genotypes, age, individual ancestry, and sex on skin color variation. Significant effects on M index variation were observed for *ASIP* genotypes ($F(2,236) = 4.37$, $P = 0.01$), ancestry ($F(1,243) = 37.2$, $P < 0.001$), and sex ($F(1,244) = 4.08$, $P = 0.05$). Subsequent analyses revealed a strong effect on M index from *ASIP* genotypes in African American females ($P < 0.001$). Our study suggests that the *ASIP* G > A polymorphism exhibits a dominant effect leading to lighter skin color and that variation in the *ASIP* gene may have been one of several factors contributing to reductions in pigmentation in some populations. Further study is needed to reveal how interactions between *ASIP* and several other genes, such as *MC1R* and *P*, predict human pigmentation.

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Introduction

Skin, hair, and eye pigmentation is due to melanin, a biopolymer produced by cells called melanocytes. Melanin plays an important role in shielding the body from ultraviolet (UV) radiation. There are two classes of melanin: eumelanin, which is associated with brown/black color, and phaeomelanin, associated with yellow/red coloration. Tyrosinase is the rate-limiting enzyme essential for the production of melanin, and the quantity of melanin synthesized is proportional to the tyrosinase activity in the cells (Robins 1991). The pathway by which eumelanin is produced is initiated by the binding of α-melanocyte stimulating hormone (α-MSH) to the melanocyte stimulating hormone receptor, also called melanocortin-1 receptor (MC1R) (MIM 155555). The binding of α-MSH to its receptor leads to increased intracellular levels of cyclic adenosine monophosphate (cAMP), increased expression of tyrosinase (TYR) (MIM 606933), which catalyzes the hydroxylation of tyrosine, and the production of eumelanin (Hunt et al. 1994). Phaeomelanin, on the other hand, is stimulated through the antagonism of α-MSH through MC1R by the agouti signaling protein (Lu et al. 1994).

In mice, the agouti signaling protein gene (*ASP*) is one of the main genes that regulates pigmentation (Suzuki et al. 1997). Loss of function mutations in mouse

ASP produce eumelanin, while gain of function mutations lead to phaeomelanin production. A variety of coat colors in mice appear as a result of these alterations (Voisey and Van Daal 2002). In humans, the ASP-encoding (MIM 600201) gene, *ASIP*, homologue to mouse *ASP*, is located on chromosome 20q11.2-q12, and encodes a 132-amino-acid protein (Wilson et al. 1995). Given that *ASIP* is conserved among species (Voisey and Van Daal 2002), it is likely that ASIP contributes to variation in human pigmentation as well. However, the precise role of ASIP in the human pigmentation pathway remains to be defined, as few studies have been conducted to date. A single-nucleotide polymorphism (SNP) in the 3′-untranslated region (UTR) of *ASIP* (noted as g.8818A > G in the literature, dbSNP# rs6058017) has been reported to be associated with dark hair and brown eyes in European Americans (Kanetsky et al. 2002) and was found to occur at different frequencies in European Americans, East Asians, African Americans and West Africans (Zeigler-Johnson et al. 2004). The proposed mechanism of action for the *ASIP* SNP involves reduced mRNA stability and premature degradation of the transcript when the G allele is present. As a consequence, binding to MC1R is biased towards α-MSH, leading to eumelanogenesis and subsequently darker pigmentation (Kanetsky et al. 2002; Zeigler-Johnson et al. 2004).

Previously, we have reported on the involvement of two other pigmentation candidate genes, namely *TYR* and the *P* gene (*OCA2*) (MIM 203200), in skin pigmentation differences between populations of European and West African descent (Shriver et al. 2003). To further explore the genetic contribution to skin color we examined the relationship between the *ASIP* 8818G allele and skin pigmentation in a sample of African Americans whose skin color was objectively measured using reflectometry.

Subjects and methods

Population

The complete sample consisted of 256 unrelated African Americans from Washington, D.C., USA. All individuals identified themselves as African American and were recruited from the Howard University campus and surrounding area. Further details about the collection of this sample are provided elsewhere (Shriver et al. 2003). Each person was measured for skin pigmentation with a DermaSpectrometer (cyberDerm, Media, Pa., USA) three times on the upper inner side (medial aspect) of each arm and these values were averaged together as described by Shriver and Parra (2000). In addition, peripheral blood was drawn by venipuncture for DNA extraction. Written informed consent was obtained from all subjects and the study was approved by the Institutional Review Board at Howard University. Finally, DNA from two unrelated chimpanzees (*Pan troglodytes*) was used in order to help determine the ancestral allele state.

Genotyping

The *ASIP* gene region containing the g.8818A > G polymorphism was amplified by PCR using 40 ng of DNA template, PCR buffer (50 mM KCl, 10 mM Tris–HCl pH 8.3, and 0.001% w/v gelatin), 2 mM MgCl, 5% DMSO, 0.2 mM dNTPs, 10 pmol/μl forward and reverse primers and 1 U of TaqGold polymerase in a 30-μl reaction. Primer sequences are described elsewhere (Kanetsky et al. 2002). The cycling protocol consisted of an initial denaturation of 12 min at 95°C, 35 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C, and a final extension of 10 min at 72°C. The 208-bp amplified fragment (10 μl of PCR product) was then digested overnight at 37°C using 5 U of *Bsr*BI restriction endonuclease, 10×*Bsr*BI buffer and sterile water up to 20 μl. All digestions were run in a 3% agarose gel. The presence of the G allele creates a *Bsr*BI cut site and consequently two fragments of 161 and 47 bp are expected.

Statistical analyses

To test for association of the *ASIP* genotype and skin color we used a general linear model where skin pigmentation was the dependent variable and the *ASIP* genotype an independent fixed factor. Since men and women from the same population have been found to exhibit significant differences in skin pigmentation (Rees 2003), we also tested models that included sex as a factor. Age was included as a covariate in some of the tests as well. Additionally, we introduced individual ancestry as a covariate in the model, as a means to control for spurious associations that may be the result of differences in ancestral proportions (admixture). Individual ancestry was estimated using a set of 34 ancestry informative markers (AIMs). Marker information can be found at the NCBI dbSNP database under the submitter handle PSU-ANTH. These markers exhibit large frequency differences between parental populations (i.e., West Africans and Europeans), and are useful to control for the presence of genetic structure due to admixture (Hoggart et al. 2003; Parra et al. 2004; Shriver et al. 2003). Ancestry estimates for each person were obtained using a maximum likelihood approach (Hanis et al. 1986). All analyses of association were performed using the SPSS program version 11.

Results

Two hundred and thirty-four individuals out of 256 were successfully typed for this SNP (Table 1). Genotype frequencies were in Hardy–Weinberg equilibrium

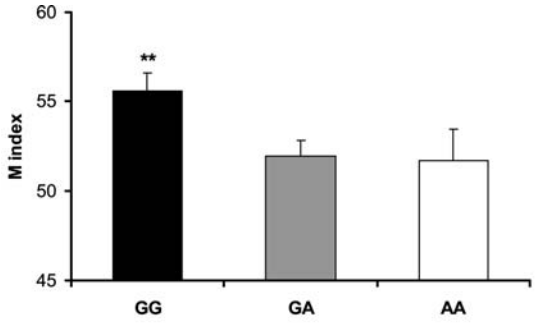
Table 1 Characteristics of the African American population (*SE* standard error)

	Total population	Females	Males	*P* value
Number	234	100	134	–
Age (SE)	32.1 (0.6)	28.8 (0.8)	34.8 (0.8)	< 0.001
M index (SE)	53.5 (0.6)	52.2 (0.9)	54.7 (0.9)	0.05
West African Admixture (SE)	78.60 (1.2)	79.51 (1.9)	77.9 (1.6)	0.53

($P > 0.05$). The frequency of the G allele among African Americans from Washington, D.C. was 66%, which is close to the frequency of 62% observed by Zeigler-Johnson et al. (2004) in African Americans from Philadelphia, Pa., USA. The G allele was observed in the homozygous state for both chimpanzee samples and therefore assumed to be the ancestral allele.

Table 1 reveals that slight, but significant differences in skin pigmentation levels were observed between males (mean M index of 54.7 ± 0.9) and females (mean M index of 52.2 ± 0.9), with men being slightly darker than women on average ($P = 0.05$). No significant differences were observed between genders with respect to average West African ancestry as estimated using the 34 AIMs (Table 1). As we have shown previously, West African ancestry was positively correlated with skin pigmentation in this population ($P < 0.001$, $R^2 = 0.129$). Women were about 6 years younger than men on average ($P < 0.001$) (Table 1). However, no relationship between age and M index was evident from the analyses ($P = 0.10$).

General linear model analyses revealed that the *ASIP* genotype was significantly associated with skin color in all models, before and after adjustment for individual ancestry and sex (Table 2). The mean M index was significantly different among the three genotypic classes, with homozygote GG individuals showing the darkest skin color (Fig. 1). Since genotype and sex exhibited a significant interaction ($P = 0.02$) we subdivided the sample according to gender and analyzed each group separately. A significant association with genotype was still evident among females ($P < 0.001$), but was absent among males ($P = 0.92$). In both groups of sexes mean M index appeared to increase with the presence of the G allele, although among men the differences between genotypic classes were not significant. However, among women, a significantly higher mean M index was seen for GG subjects compared with GA and AA females combined (GG mean M index = 56.5; GA/AA, mean M index = 49.6, $P < 0.001$).



Note.- Differences in M index between genotypes were tested using ANOVA with a Bonferroni correction, ** p<0.05.

Fig. 1 Mean M index by *ASIP* genotype among African Americans. Differences in M index between genotypes were tested using ANOVA with a Bonferroni correction, $^{**}P < 0.05$

Discussion

Given the role ASIP plays in the switch from eumelanin to phaeomelanin production, it is one of many strong candidates to explain interindividual variation in human pigmentation. Previous analyses of the *ASIP* gene have implicated the 3′UTR g.8188A > G SNP with hair and eye pigmentation phenotypes, but the ancestral state and its relationship to skin pigmentation has never been studied. Our results from genotyping the chimpanzee suggest that the ancestral state at position 8188 of the *ASIP* gene 3′UTR most likely is the G allele. The G allele is more frequent in darker-skinned populations like West Africans and their descendant populations, such as African Americans (Zeigler-Johnson et al. 2004; this study). Since the ancestors of modern humans originated in Africa and later migrated outwards to Asia and Europe, it makes evolutionary sense that the ancestral nucleotide shows its highest frequencies in the sub-Saharan Africa. It would be interesting to determine the frequency of the *ASIP* SNP in distinct non-African populations such as Melanesians and other

Table 2 Statistical models tested using melanin (M) index as the dependent variable, and *ASIP* genotype, sex and/or ancestry as independent factors/covariates

Model	*F* (*df*; error)	R^2	*P* value			
			ASIP	Sex	Ancestry	*ASIP*×sex
ASIP	4.4 (2; 236)	0.03	0.01	–	–	–
Sex	4.1 (1; 244)	0.01	–	0.05	–	–
Ancestry	37.2 (1; 243)	0.13	–	–	< 0.001	–
Sex + ancestry	20.9 (2; 240)	0.14	–	0.03	< 0.001	–
ASIP + sex	3.2 (5; 228)	0.05	0.007	0.22	–	0.07
ASIP + ancestry	14.8 (3; 231)	0.15	0.02	–	< 0.001	–
ASIP + sex + ancestry	9.5 (6; 226)	0.18	0.005	0.12	< 0.001	0.02

dark-skinned southeast Asians. We have recently found that the *ASIP* 8188G allele has a very low frequency of 1.5% in indigenous Nahuas from the state of Guerrero, Mexico, whose average M index is 47.1 ± 0.48 (Bonilla 2003). We also note that in a population sample of Puerto Ricans the frequency of the G allele reached 37.5%, while their mean M index was only of 36.9 ± 0.76 (Bonilla et al. 2004). These results suggest that dark skin is due to several factors, such as the total melanin content and the proportion of phaeomelanin to eumelanin in the skin. Thus, it is likely that different sets of genes are implicated in skin pigmentation in different populations. In fact, experiments conducted by Alaluf et al. (2002) showed that the amount of light, alkali soluble melanin (namely phaeomelanin) decreases as the skin pigmentation becomes darker. However, for Indians from the subcontinent of India, their skin exhibited a relatively high concentration of phaeomelanin, while also possessing significant levels of eumelanin, making the total concentration of epidermal melanins comparable with that of Africans.

In addition, we have examined the effect that the A > G polymorphism has on skin pigmentation in African Americans. Although the SNP had been previously associated with dark hair and eyes in European-Americans (Kanetsky et al. 2002), little was known about its correlation with darker skin color in a population with diverse levels of European and West African ancestry. Our results reveal that, in fact, the presence of the ancestral G allele is associated with a higher M index in African Americans, even after correcting for individual admixture. Because there are differences in ancestry among the individuals in this population (see Shriver et al. 2003), the association of SNPs with phenotypic traits that differ among ancestral populations may be the result of population stratification and thus may not underlie a causative effect (Halder and Shriver 2003; Kittles et al. 2002; Shriver and Kittles 2004; Shriver et al. 2003). The addition of individual admixture as a covariate in the model was used to correct for confounding by ancestry. It is worth noting, however, that even though the number of AIMs used in this study to infer ancestry is appropriate to detect population stratification, more AIMs will be needed to provide individual ancestry estimates with lower associated standard errors.

Interestingly, the effect of the *ASIP* variant does not appear to be similar for males and females, as the difference in pigmentation between both sexes is not the same for all genotypic categories. Nevertheless, it should be noted that analyzing each sex separately leads to a reduction in statistical power due to a smaller sample size. It is apparent that the *ASIP* genotype, gender, and ancestry, all influence pigmentation, although at different levels, when tested separately (Table 2). The sexual dimorphism observed in the relationship between ASIP and skin color clearly needs to be examined. Earlier, Voisey et al. (2002) reported that *ASIP* gene expression in adipose tissue was associated with BMI in opposite ways in men and women and suggested that this difference may be related to the interaction with sex steroids. Further studies are necessary to clarify these findings.

In a previous article we have shown that *TYR* and *OCA2*, two other pigmentation candidate genes, also had an appreciable effect on skin color in African Americans (Shriver et al. 2003). In contrast, the *MC1R* gene did not seem to have an impact on skin pigmentation in African Americans, even though it has been linked to skin color variation in populations of European descent (Rees 2003). It is likely that several genes influence human pigmentation, possibly each gene functioning differently in different populations to contribute small or modest effects on the phenotype. For example, recently, ASIP was found to downregulate the expression of tyrosinase-related protein 1 (TYRP1) (MIM 115501), a component of the melanogenic pathway in the mouse, and five other novel candidate genes, in human melanoma cells (Voisey et al. 2003). In the Voisey et al. study, tyrosinase was not affected by ASIP. These observations make it clear that further investigations are needed to determine which genetic interactions predict human pigmentation.

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