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ORIGINAL INVESTIGATION

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Interaction between the melanocortin-1 receptor and *P* genes contributes to inter-individual variation in skin pigmentation phenotypes in a Tibetan population

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Abstract The melanocortin-1 receptor (MC1R) and *P* gene product are two important components of the human pigmentary system that have been shown to be associated with red hair/fair skin and cause type II oculocutaneous albinism, respectively. However, their contribution to inter-individual variation at the population level is not well defined. To this end, we genotyped 3 single nucleotide polymorphisms (SNPs) in the *MC1R* gene (Arg67Gln, Gln163Arg, Val92Met) and 2 SNPs in the *P* gene (IVS13–15 and Gly780Gly) in 184 randomly ascertained Tibetan subjects, whose skin color was measured as a quantitative trait by reflective spectroscopy. Single locus analyses failed to demonstrate an association between any of the 5 SNPs and skin pigmentation. However, when an epistatic model was applied to the data, a significant gene-gene interaction was identified between Val92Met in MC1R and IVS13–15 in the *P* gene (F=2.43; P=0.0105). We also discuss the possible mechanisms of how gene interactions arise in signal transduction pathways.

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Introduction

Skin pigmentation is one of the most visible and diverse of all human phenotypes and its study can address many practical problems ranging from understanding skin cancer susceptibility (Palmer et al. 2000) to questions regarding human evolutionary history (Rana et al. 1999). Human pigmentation is primarily determined by the pigment melanin, which is synthesized in the melanosome (an organelle contained within melanocytes). There are two predominant forms of mammalian melanin: (1) the black/brown eumelanin, and (2) the yellow/red pheomelanin. The initial biochemical steps in the synthesis of eumelanin and pheomelanin are identical and include the oxidation of tyrosine to dopaquinone by the enzyme tyrosinase. Dopaquinone is the last common precursor of eumelanin and pheomelanin, whose fate is largely determined by the signaling state of the melanocortin-1 receptor (MC1R).

The MC1R is a seven-pass G-protein-coupled receptor that is activated by melanocortins, such as the melanocyte-stimulating hormone (αMSH) (reviewed by Cone et al. 1996), and is antagonized by the human homolog of the mouse agouti locus (Suzuki et al. 1997). Activation of MC1R leads to an increase in intracellular cAMP, which ultimately mediates the expression of enzymes important in the biosynthesis of eumelanin. One of these enzymes is encoded by the *P* gene, and is a melanosomal integral membrane protein, whose function may be to stabilize the multi-protein complex important in eumelanin synthesis (Lamoreux et al. 1995). Thus, the signaling state of MC1R helps to determine the ratio of eumelanin to pheomelanin (reviewed by Abdel-Malek et al. 1999). When MC1R is inactive, dopaquinone is preferentially converted into pheomelanin. Overall, the observed pigmentation of an individual is the culmination of the size, shape, distribution, and chemical composition of melanin in the keratinocytes and hair (Rana et al. 1999).

Although many of the molecular details of pigmentation are becoming clear, relatively little is understood about

the genes responsible for differences in skin color among individuals within or between populations. Genetic variation in several genes has been described that lead to relatively rare pigmentation phenotypes. For example, at least 32 different mutations in the *P* gene cause type II oculocutaneous albinism (OCA2) (reviewed by Oetting and King 1999). While considerable information about the pigmentary system has been gleaned by identifying and studying the mutations that underlie these rare clinical conditions, the genes that contribute to inter-individual variation in skin color within the population-at-large remain obscure. Thus far, only MC1R has been implicated in normal human skin color variation, and particular variants have been found to be associated with red-haired and/or pale-skinned individuals of European descent (Valverde et al. 1995; Box et al. 1997; Rees and Flanagan 1999; Flanagan et al. 2000).

The purpose of this study was to investigate the contribution of the *MC1R* and *P* genes to inter-individual variation in skin pigmentation in a Tibetan population.

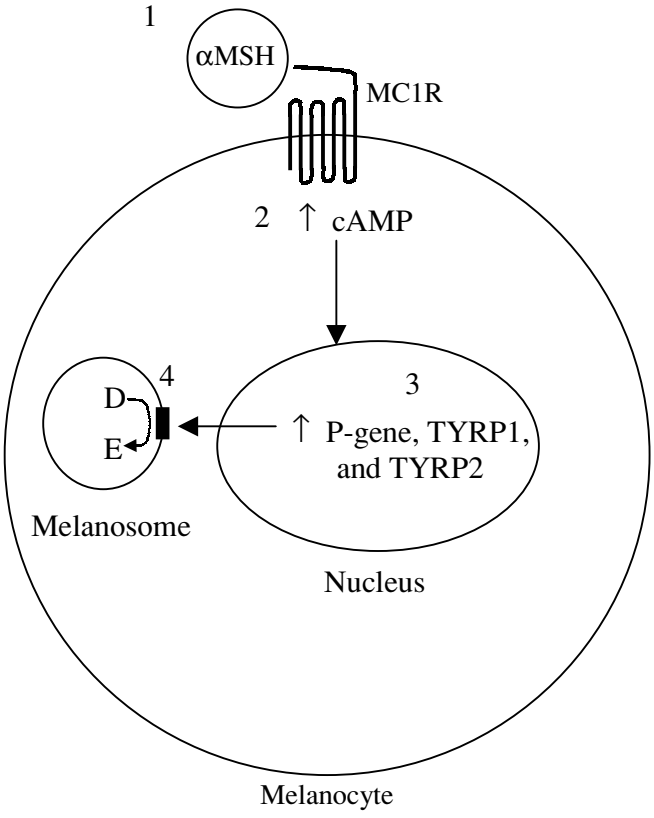

Fig. 1 Interaction of MC1R and the *P* gene product in eumelanin biosynthesis. Binding of αMSH to MC1R (*1*) leads to an increase in intracellular cAMP concentration (*2*) and initiates a signal transduction cascade resulting in the expression of eumelanin-promoting enzymes, such as the *P* protein, tyrosinase-like protein 1 (*TYRP1*) and tyrosinase-like protein 2 (*TYRP2*) (*3*). These enzymes are then directed to the melanosome membrane and form a macromolecular complex, indicated by the black rectangle (*4*), which catalyzes the conversion of dopaquinone (*D*) into eumelanin (*E*)

Materials and methods

Subjects

The Tibetan sample consisted of 184 randomly ascertained students, age 12 to 19 years old, from the Shanghai Tibetan School in Shanghai, China. The average age (±SE) of individuals was 14.1± 1.01, and the sample consisted of 55% females and 45% males. Skin pigmentation levels for each individual were measured between September and November 1998. Informed consent was obtained from each study participant and group consent for minors was obtained from the appropriate authorities at the Shanghai Tibetan school. All rules and regulations as outlined by Fudan University and the Chinese Medical Academy were strictly followed.

Measuring skin pigmentation levels

Skin pigmentation was measured as a quantitative trait by the Photovolt ColorWalk reflectometer (UMM Electronics, Indianapolis, Ind.), which uses photodiode arrays to measure the intensity of particular wavelengths of light. We measured pigmentation by the L*, or the lightness-darkness measure, which is a standard color parameter (where higher values of L* indicate lighter skin). Three measurements were taken in the unexposed area of the upper inner arm and the average of these measurements was used in the analyses reported here. More specific details of how skin pigmentation was measured can be found elsewhere (Shriver and Parra 2000).

SNP genotyping

We genotyped three previously described SNP markers in *MC1R* (Valverde et al. 1995; Box et al. 1997) and two SNPs in the *P* gene (Lee et al. 1995). The proteins encoded by the *MC1R* and *P* genes participate in a common biochemical pathway, as shown in Fig.1. The three *MC1R* SNPs all occur in coding regions and result in non-synonymous amino acid substitutions (Arg67Gln, Gln163Arg, Val92Met). Of the two *P* gene SNPs genotyped, one occurs in the coding region and results in a synonymous amino acid substitution (Gly780Gly), while the other occurs 15 bp upstream of the acceptor splice site in intron 13 (IVS13–15). Details of these markers can be found in Table 1. PCR was performed in a GeneAmp 9700 (Applied Biosystems, Foster City, Calif.) and consisted of the following thermocycles: 95° for 5 min, followed by 30 cycles of 95° for 30 s, 55° for 30 s, 72° for 30 s, and a final extension at 72° for 5 min. PCR reactions were performed in 25 µl reactions consisting of 25 ng genomic DNA, 1.5 mM $MgCl_2$, 2.5 µl 10×PCR buffer (Gibco BRL, Rockville, Md.), 0.1 mM dNTPs, 0.04 µM of each primer and 2.5 units Taq polymerase (Gibco BRL, Rockville, Md.). Primer sequences were as follows: Arg67Gln (5'-GGAAGAACTGTGGGGACCTGGAG-3' and 5'-TAAGGAACTGCCCAGGGTCACAC-3'), Gln163Arg (5'-CAGGCCAAGAGCGTCCTA-3' and 5'-TGCCACTCTGTGAACAGCAA-3'), Val92Met (5'-CATCTGAGTGCAAGATAAAAAGGA-3' and 5'-CCCACCCCCAAATCATCTAT-3'), IVS13–15 (5'-AATCTCTGGGTTGCATGTGG -3' and 5'-CTTCCTCAGCTCTTGGTTGG -3'), and Gly780Gly (5'-ATCAAATCAAAGCCTGTGAG -3' and 5'-ACCTGAAAAATTCCATGAAG -3'). Alleles were scored by restriction enzyme digestions, which were performed in a final volume of 25 µl, as recommended by the suppliers, for a period of between 4 and 24 h. Specifically, Arg67Gln, Gln163Arg, Val92Met, IVS13–15, and Gly780Gly were digested by *Msp*I, *Sac*I, *Nsp*I, *Mbo*II, and *Mae*III, respectively. All restriction enzymes were purchased from New England Biolabs (Beverly, Mass.). Digestion products were resolved by agarose-gel electrophoresis.

Statistical methods

In all of the statistical methods discussed below, L* values were adjusted for sex by regressing L* on sex and subtracting the sex

term from the original unadjusted L* measures. Allele frequencies were estimated by gene-counting, and significant departures from Hardy-Weinberg equilibrium (HWE) were tested by either a chi-square test or permutation method (Weir 1996), where appropriate. Single locus analysis of association between SNPs and pigmentation levels (i.e., L*) were investigated by multiple linear regression with genotype, age and sex used as independent variables.

Two-locus tests of association were performed by a method similar to a two-way factorial analysis of variance with a test for interaction of main effects. Briefly, consider two trait loci, Q and q, each with two alleles denoted as 1 and 2. Allele frequencies at the two trait loci Q and q are P_{Q1} and P_{Q2} and P_{q1} and P_{q2}, respectively. The two-locus genotypic values G_{ijkl} are the average phenotypic values for individuals who have the genotypes Q_iQ_j and q_kq_l at the trait locus Q and q, respectively. Let μ_o be the overall mean genotypic value and a_Q, a_q, d_Q, and d_q be the additive and dominance genotypic values at locus Q and q, respectively. The four independent epistatic genotypic values: additive × additive, additive × dominance, dominance × additive, and dominance × dominance are denoted by e_{AA}, e_{AD}, e_{DA}, and e_{DD}, respectively. The two-locus genotypic value G_{ijkl} can be expressed as a function of the additive and dominance genotypic values at the single locus and the epistatic genotypic values as:

$$G_{1111} = \mu_o + \alpha sex + a_Q + a_q + e_{AA},\ G_{1112} = \mu_o + \alpha sex + a_Q + d_q + e_{AD},$$
$$G_{1122} = \mu_o + \alpha sex + a_Q - a_q - e_{AA},\ G_{1211} = \mu_o + \alpha sex + d_q + a_q + e_{DA},$$
$$G_{1212} = \mu_o + \alpha sex + d_Q + d_q + e_{DD},\ G_{1222} = \mu_o + \alpha sex + d_q - a_q - e_{DA},$$
$$G_{2211} = \mu_o + \alpha sex - a_Q + a_q - e_{AA},\ G_{2212} = \mu_o + \alpha sex - a_Q + d_q + e_{AD},$$
$$G_{2222} = \mu_o + \alpha sex - a_Q - a_q + e_{AA}$$

Let S_F^2 and S_R^2 be the residual variances estimated from fitting the observed data to the full model (described above) and the reduced model (e.g., $e_{AA} = 0$, $e_{AD} = 0$, $e_{DA} = 0$, and $e_{DD} = 0$), respectively. The optimal fit and parameters were computed by the standard least squares method (i.e., minimizing the sum of square of the errors between the observed adjusted L* values and those predicted by the assumed model). The overall level of epistasis can be tested for statistical significance by an *F*-test:

$$F_{ep} = \frac{n-9}{4}\frac{(S_R^2 - S_F^2)}{S_F^2}$$

with 4, n-9 degrees of freedom (Cheverud and Routman 1995; Graybill 1976).

Results

Single locus analyses

All markers were in HWE, except for the Gly780Gly polymorphism of the *P* gene (*P*=0.009). Table 1 shows the *F*-statistic and *P*-values for the single locus analyses. Individually none of the 5 SNPs demonstrate an association with skin pigmentation levels, although IVS13–15 of the *P* gene approaches significance (*F*=3.076, *P*=0.081). Sex was significantly related to pigmentation levels (*F*=23.8, $P=4\times10^{-9}$).

Table 1 Results of single locus association analysis

Gene	Polymorphism	SNP type	SNP frequency	*F*	*P*-value
MC1R	Arg67Gln	C/T	0.992/0.008	0.014	0.907
	Gln163Arg	G/A	0.758/0.242	1.092	0.297
	Val92Met	C/T	0.955/0.045	0.717	0.398
P	IVS13–15	C/T	0.867/0.132	3.076	0.081
	Gly780Gly	C/T	0.965/0.035	0.036	0.851

Table 2 Results of gene-gene interaction model

MC1R gene	*P* gene	F_{ep}	*P*-value
Gln163Arg	IVS13–15	0.721	0.7036
Gln163Arg	Gly780Gly	0.9609	0.4803
Val92Met	IVS13–15	2.434	0.0105
Val92Met	Gly780Gly	0.4054	0.9422

Two locus analyses

There is considerable evidence for interactions between components of the pigmentary system in model organisms (Moore et al. 1990; Bennett et al. 1998). Therefore, we also analyzed the five SNPs for association with pigmentation levels under a gene-gene interaction model described above. The F_{ep}-test was applied to *MC1R* and *P* gene SNPs, and the results are summarized in Table 2. The Arg67Gln polymorphism of *MC1R* was excluded from the interaction analysis due to the very small minor allele frequency. Although the Val92Met and IVS13–15 variants are not individually associated with skin color, the two loci do show evidence of interacting to impact pigmentation levels (F_{ep}=2.434, nominal *P*-value <0.0105).

Figure 2 shows the single and two-locus genotypic means for the Val92Met and IVS13–15 variants of the *MC1R* and *P* genes, respectively. The single locus genotypic means are relatively consistent across genotypes. However, individuals with the Val92Met T allele show a non-significant trend towards lighter skin color. For the two-locus genotypic values, it is obvious that the mean of a particular polymorphism depends upon the genetic context in which it occurs. For example, mean L* genotypic values for the Val92Met variant decrease with increasing copies of the IVS13–15 T allele. In fact, the largest two-locus genotypic mean occurs in CT/CC individuals, which is consistent with the single-locus trends. However, due to the small number of observations for some two-locus genotypes caution must be taken in interpreting the data and this result should be viewed as intriguing, subject to verification in larger data sets. More generally, our study highlights the need for large sample sizes in detecting gene-gene interactions.

Discussion

A great challenge in contemporary genetics research is to elucidate the molecular and genetic architecture of quantitative traits. Skin pigmentation is an ideal model phenotype to begin to understand quantitative traits because it is easily measured, has important implications in evolutionary and medical studies, and many of the molecular de-

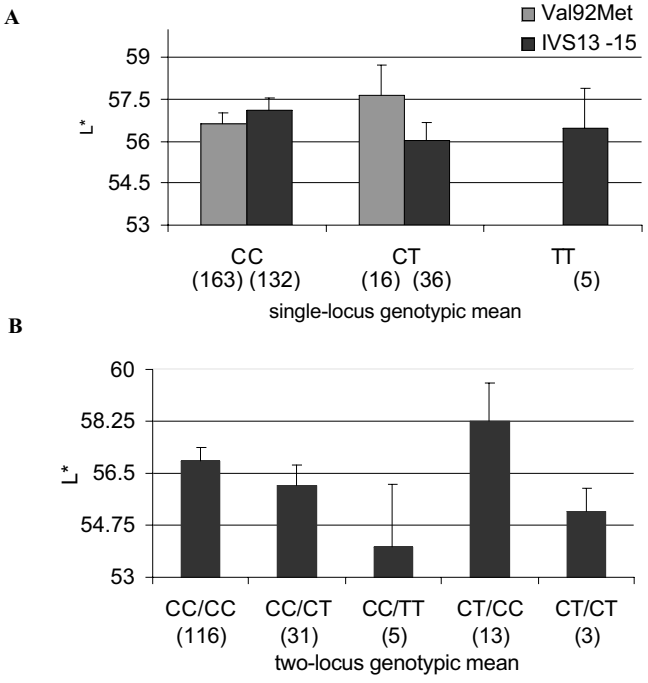

Fig. 2A, B Genotypic means of Val92Met and IVS13–15 variants. **A** Single locus genotypic means ± SD. Due to the low frequency of the Val92Met T allele, no homozygotes were observed. **B** The two-locus genotypic means ± SD are denoted as Val92Met/IVS13–15 genotype. The number of individuals is noted in parenthesis below each genotype

tails of the pigmentary system are becoming clear. In this report, we have provided the first evidence of a gene-gene interaction between the *MC1R* and *P* genes, which contributes to inter-individual variation in skin pigmentation of a Tibetan population.

To date, the functional ramifications of the Val92Met polymorphism of MC1R on inter-individual variation in skin pigmentation levels remains ambiguous. Functional analysis of the Val92Met variant revealed an approximately 5-fold lower affinity for its ligand, αMSH, compared to wild-type MC1R (Xu et al. 1996). Thus, it is reasonable that we observe individuals who have a Met allele to have lighter skin (Fig. 2). However, in a heterologous expression system no differences were observed between the Val and Met alleles' ability to activate adenyl cyclase (Koppula et al. 1997). These two results are not necessarily conflicting, as it may be that the Val92Met variant only affects ligand binding, but plays no role in stimulating adenyl cyclase.

Furthermore, results of previous association analyses have been contradictory, as some studies support a physiological role for Val92Met (Valverde et al. 1995), while others do not (Flanagan et al. 2000; Box et al. 1997). These incongruent results can be rectified in the context of gene-gene interactions. For example, if the effect of Val92Met depends on the presence of alleles at other loci, association studies considering only marginal effects will have limited power in detecting its contribution to skin pigmentation levels. It is not immediately clear whether the IVS13–15 polymorphism is of functional significance (for instance affecting splicing), or alternatively, is in linkage disequilibrium with an unidentified functional variant.

Finally, it is interesting to consider the molecular basis for the observed interaction. Although the *MC1R* and *P* genes do not physically interact, they are connected through the involvement of their encoded proteins in an intracellular signaling pathway (see Fig. 1). A fundamental characteristic of pathways that transduce extracellular signals into intracellular ones is amplification of the original signal (Alberts et al.1994). For example, binding of one αMSH molecule to MC1R results in the production of many cAMP molecules. We hypothesize that the observed interaction between variation in the *MC1R* and *P*-genes is a consequence of the mechanism of intracellular signaling. If this hypothesis is generally correct, it may have important implications in the design and interpretation of association studies involving components of signaling pathways.

In conclusion, even quantitative trait loci for an "ideal" quantitative trait, such as pigmentation, will often manifest complex genotype-phenotype relationships, such as gene-gene interactions and pleiotropy (Flanagan et al. 2000). Future studies will be required to replicate our findings across populations, extend our analyses to consider intra-locus interactions, and to elucidate the molecular basis of this observation.

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