Short Communication

P Gene as an Inherited Biomarker of Human Eye Color[1]

Timothy R. Rebbeck,[2] Peter A. Kanetsky, Amy H. Walker, Robin Holmes, Allan C. Halpern, Lynn M. Schuchter, David. E. Elder, and DuPont Guerry

Departments of Biostatistics and Epidemiology [T. R. R., P. A. K., A. H. W., R. H.] and Pathology and Laboratory Medicine [D. E. E.], Division of Hematology/Oncology [L. M. S., D. G.], and The Melanoma Program of Cancer Center [T. R. R., P. A. K., L. M. S., D. E. E., D. G.], University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, and Memorial Sloan-Kettering Cancer Center, New York, New York 10021 [A. C. H.]

Abstract

Human pigmentation, including eye color, has been associated with skin cancer risk. The *P* gene is the human homologue to the mouse pink-eye dilution locus and is responsible for oculocutaneous albinism type 2 and other phenotypes that confer eye hypopigmentation. The *P* gene is located on chromosome 15q11.2-q12, which is also the location of a putative eye pigmentation gene (*EYCL3*) inferred to exist by linkage analysis. Therefore, the *P* gene is a strong candidate for determination of human eye color. Using a sample of 629 normally pigmented individuals, we found that individuals were less likely to have blue or gray eyes if they had *P* gene variants *Arg305Trp* ($P = 0.002$), *Arg419Gln* ($P = 0.001$), or the combination of both variants ($P = 0.003$). These results suggest that *P* gene, in part, determines normal phenotypic variation in human eye color and may therefore represent an inherited biomarker of cutaneous cancer risk.

Introduction

Human pigmentation is a complex trait that is likely to be determined by inherited genotypes. At least three genes have been inferred from genetic linkage analysis to explain normal phenotypic variability in human eye color (1), including *EYCL1* (*GEY*; chromosome 19), *EYCL2* (*BEY1*; chromosome 15), and *EYCL3* (*BEY2*; chromosome 15). To date, none of these genes has been isolated. Eiberg *et al.* (1) suggested that the *P* gene (chromosome 15q11.2-q12), the human homologue of the mouse pink-eye dilution locus, is a candidate for *EYCL3*. The P protein may stabilize (2) or traffic (3) melanosomal proteins such as tyrosinase, regulate melanosomal pH (4), or serve as a melanosomal tyrosine transporter (3, 5). Altered P protein may therefore affect pigmentation characteristics via altered melanosomal tyrosine or tyrosinase bioavailability or function. Mutations in the *P* gene are associated with OCA2, the most common type of human albinism (6). Hypopigmentation of the skin, hair, and eyes in Prader-Willi Syndrome has also been associated with *P* gene deletions (7). These data suggest that allelic variation in the *P* gene may be associated with normal variability in human eye color.

Materials and Methods

To evaluate whether the *P* gene is associated with human eye color in nonalbino individuals representing the normal phenotypic range of human eye color, we studied 629 Caucasian individuals ascertained at the Pigmented Lesion Clinic at the Hospital of the University of Pennsylvania between 1997 and 2000 on whom we had both eye color data and at least one *P* genotype. Study participants were selected without regard to pigmentation type, knowledge of family history of MM, or other MM risk factors. These individuals included 127 with DN, 200 with a single incident primary invasive MM and no history of DN, 133 MM cases with one or more DN, and 169 controls with no history of MM or DN who were friends or nonbiological relatives of MM/DN cases. All study participants signed an informed consent form that was approved by the Committee for the Study of Human Subjects at the University of Pennsylvania. Each participant donated a buccal swab (Medical Packaging Corporation, Camarillo, CA) that was processed to obtain genomic DNA using the method described in Walker *et al.* (8). PCR analysis was used to determine genotypes for two *P* gene variants (Fig. 1). Of 629 individuals, 27 individuals were missing *Arg305Trp* genotypes only, and 17 individuals were missing *Arg419Gln* genotypes because of genotype failures. In the present sample of Caucasian controls, the *305Trp* and *419Gln* allele frequencies were 6.8 and 8.2%, respectively.

Eye color was determined upon clinical examination by a single research nurse using the following categories: blue; gray; green; hazel; light brown; dark brown; and black. Participants also completed a standardized questionnaire that asked self-assessed eye color. The correlation between self-assessed and clinician-assessed eye color was 93%, and there was 100% concordance between self- and clinician-assessed eye color once categorizations were made for analyses. Therefore, analyses were undertaken using categorized exam-determined eye color. Categories of eye color used in analysis were blue/gray ($n = 255$, 40.5%), green/hazel ($n = 170$, 27.0%), and brown/black ($n = 204$, 32.4%). Contingency table analysis using the χ^2 test was undertaken to evaluate the relationship of *P* gene variants and eye color. Unconditional logistic regression analysis was used to estimate the OR effect of *P* gene variants on eye color adjusted for diagnostic outcomes of DN and/or MM. These adjustments were undertaken to remove potential confounding relationships between disease status, *P* gene variants, and eye color.

Received 12/31/01; revised 4/23/02; accepted 5/4/02.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported by USPHS Grant P01-CA75434.
[2] To whom requests for reprints should be addressed, at Department of Biostatistics and Epidemiology, University of Pennsylvania School of Medicine, 904 Blockley Hall, 423 Guardian Drive, Philadelphia, PA 19104. Phone: (215) 898-1793; Fax: (215) 573-2265; E-mail: trebbeck@cceb.med.upenn.edu.
[3] The abbreviations used are: OCA2, oculocutaneous albinism type 2; MM, melanoma; DN, dysplastic nevi; OR, odds ratio.

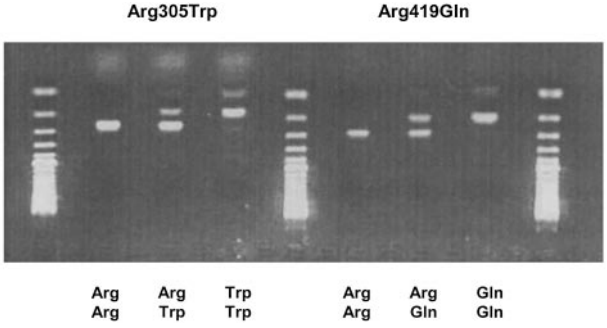

Fig. 1. Gel electrophoresis of *P* gene variants. *Arg305Trp* and *Arg419Gln* alleles were determined by using a PCR-based assay that modified the protocol of Lee *et al.* (11). The two primers used to amplify exon 9 containing Arg305Trp were: P9for, 5′-AGAGGGAGGTCCCCTAACTG-3′ and P9rev, 5′-ATCTCAAGCCTCCCTGACTG-3′. The PCR reaction mix consisted of 5 μl of 10X Perkin-Elmer Buffer II [500 mM KCl, 100 mM Tris-HCl (pH 8.3), 3.0 μl of 25 mM $MgCl_2$, 0.5 μl of 10 mM deoxynucleotide triphosphate, 5 μl each of the primers at 5-μM concentration, 8 μl of template DNA, and 0.4 μl of Platinum Taq Polymerase in 23.1 μl of double-distilled water for a reaction volume of 50 μl]. The PCR temperature profile included 1 cycle of 94°C for 3 min. This was followed by 30 cycles of 94°C for 40 s, 65°C for 1 min, and 72°C for 2 min, ending with an elongation step at 72°C for 5 min. The amplification products were then digested with *Msp*I (New England Biolabs) in a reaction consisting of 20 μl of PCR product, 3 μl of New England Biolabs buffer no. 2, 6.5 μl of double-distilled water, and 0.5 μl *Msp*I at 37°C for 3 h. The two primers used to amplify exon 13 containing Arg419Gln were: P13for, 5′-GCCTCTGTTCTACGAGCCTG-3′ and P13rev, 5′-TGCCTGCCAGAACCTGGCCGCAA-3′. The PCR reaction mix was as above, except for use of 5 μl of 25 mM $MgCl_2$. The PCR temperature profile included 1 cycle of 95°C for 3 min. This was followed by 30 cycles of 95°C for 40 s, 63°C for 1 min, and 72°C for 2 min, ending with an elongation step at 72°C for 5 min. The amplification products were then digested with *Ava*I (New England Biolabs) in a reaction consisting of 20 μl of PCR product, 3 μl of New England Biolabs buffer no. 4, 6 μl of double-distilled water, and 1 μl of *Ava*I at 37°C for 3 h. Visualization of the digestion products was accomplished on a 2% agarose gel with ethidium bromide.

Results and Discussion

As shown in Table 1, there was an increased proportion of *305Trp* alleles in individuals with brown/black eyes compared with those with lighter eyes ($\chi^2_2 = 13.13$; $P < 0.001$), and an increased proportion of *419Gln* alleles in individuals with green/hazel eyes compared with others ($\chi^2_2 = 18.69$; $P < 0.001$). Considering both variants simultaneously, there was an increase in the frequency of *305Trp* or *419Gln* alleles among individuals with green, hazel, brown, or black eyes compared with those with blue or gray eyes ($\chi^2_2 = 14.68$; $P < 0.001$). These results suggest that carriers of variant *P* gene alleles are significantly less likely to have blue eyes than individuals who carry no variants at this locus. In our data, the *Arg305Trp* and *Arg419Gln* variants are in linkage equilibrium with one another ($\chi^2_1 = 0.49$; $P = 0.483$). Thus, both variants studied here could independently influence eye color.

We also evaluated whether *P* gene alleles were associated with hair color. There was a marginally significant difference in the proportion of individuals with the *Arg305Trp* variant in individuals with red/blond (9.4%) *versus* brown/black (16.0%) hair ($\chi^2_1 = 4.18$, $P = 0.041$). The unadjusted OR for red/blond hair as predicted by *Arg305Trp* variant genotypes was 0.55 (95% confidence interval: 0.30–0.98). The magnitude of this association was not substantially changed but became statistically nonsignificant after adjustment for disease status (OR = 0.58, 95% confidence interval: 0.32–1.04). Our data suggest an association between *P* gene variants and hair color may exist, but our sample appears to have limited statistical power to address this question. However, the associations observed here suggest that *Arg305Trp* and possibly *Arg419Gln* are associated with darker pigmentation.

Finally, we adjusted our eye color analyses for hair color to evaluate whether other pigmentation phenotypes were confounding the relationship of *P* gene and eye color (results not shown). The differences between the adjusted and unadjusted OR estimates were small in all cases, and the adjustment made no difference in the inferences. Therefore, the relationship between the *P* gene variants and eye color appears to be largely independent of other major pigmentation phenotypes. Eye color is correlated with other pigmentation phenotypes, including hair color, skin type, and freckling, in a presumably complex manner. Our simple multivariable adjustments are unlikely to have properly accounted for this complexity. Therefore, additional studies should be done to simultaneously consider the complex relationships of *P* gene variants, UV exposure, and other pigmentation characteristics.

The functional significance of the *P* gene alleles studied here is not well understood. Indeed, controversy remains about the function of the P protein itself (2–5). *Arg305Trp* may be in linkage disequilibrium with a pathogenic 2.7 deletion mutation in OCA2 subjects (9, 10), which further complicates the relationship of *Arg305Trp* and pigmentation in OCA2 subjects. However, there is limited information that supports a functionally relevant role for *Arg305Trp* in determining human pigmentation. Most *P* gene variants that confer OCA2 risk occur outside the 12 transmembrane domains of the gene, whereas most variants not associated with OCA2 fall within a transmembrane domain. The *Arg305Trp* variant falls outside of the transmembrane domains. Most previously reported *P* gene variants not associated with OCA2 have a higher frequency in people of Caucasian descent relative to that in people of African descent (9, 11). We report a *305Trp* allele frequency of 7% in Caucasian controls with no history of MM or DN. Lee *et al.* (11) reported the *305Trp* frequency to be 90% in non-OCA2 African Americans and 17% in non-OCA2 Caucasians (11). This result is consistent with the hypothesis that *305Trp* is associated with darker pigmentation. In contrast, Kerr *et al.* (9) reported no substantial difference when comparing *305Trp* allele frequencies in non-OCA2 subjects of Caucasian or African descent. To resolve whether *Arg305Trp* and other non-OCA2 *P* gene variants influence human pigmentation, additional studies are required that describe the functional significance of the *P* gene alleles, simultaneously consider multiple *P* gene variants or haplotypes, consider other genotype combinations in addition to the simple recessive model presented here, and relate these variants to relevant pigmentation and other phenotypes.

Lee *et al.* (11) reported the existence of 28 germ-line variants in the *P* gene that were not associated with OCA2, 23 of which were silent synonymous substitutions or occurred in introns. Kerr *et al.* (9) reported additional alleles not associated with OCA2, all of which were also silent or intronic. Of the missense variants reported to date that are not associated with OCA2, *His615Arg* was not found to be polymorphic in Caucasians (11). We genotyped 312 individuals for *Leu440Phe*, which is located in a potentially functionally important region of the *P* gene (11) and found only two 440Phe variants corresponding to an allele frequency of 0.3%. Therefore, *His615Arg* and *Leu440Phe* were not studied additionally here. *Ile722Thr* has a frequency of 26% (11) and could be considered in future studies of human pigmentation. As additional *P* gene variants are identified, they could also be considered in studies that extend the present results if they are sufficiently polymorphic and if functional relevance could be assigned to them.

Table 1 *P* gene variants and eye color

	Arg305Trp		*Arg419Gln*		Combined variants	
	Arg/Arg	*Arg/Trp* or *Trp/Trp*	*Arg/Arg*	*Arg/Gln* or *Gln/Gln*	Arg only	Any Trp or Gln
Blue/gray	221 (90.2%)	24 (9.8%)	221 (89.1%)	27 (10.9%)	185 (78.7%)	50 (21.3%)
Green/hazel	142 (88.2%)	19 (11.8%)	121 (72.9%)	45 (27.1%)	101 (63.5%)	58 (36.5%)
Brown/black	154 (78.6%)	42 (21.4%)	166 (83.8%)	32 (16.2%)	122 (64.2%)	68 (35.8%)
χ^2 ($df = 2$)	13.13 ($P < 0.001$)		18.69 ($P < 0.001$)		14.68 ($P < 0.001$)	
OR[a]	1.12 (0.59–2.15)		3.00 (1.76–5.11)		2.02 (1.28–3.18)	
OR[b]	2.48 (1.43–4.30)		1.65 (0.85–2.88)		2.08 (1.34–3.23)	
OR[c]	1.78 (1.07–2.96)		2.45 (1.39–3.62)		2.05 (1.39–3.01)	

[a] OR comparison of blue/gray *versus* green/hazel adjusted for presence/absence of MM and presence/absence of DN.
[b] OR comparison of blue/gray *versus* brown/black adjusted for presence/absence of MM and presence/absence of DN.
[c] OR comparison of blue/gray *versus* green/hazel/brown/black adjusted for presence/absence of MM and presence/absence of DN.

Light (particularly blue) eye color has been reported to be a risk factor for MM. In a combined analysis of 10 studies of MM risk factors, Bliss *et al.* (12) reported that blue eye color remained a significant independent predictor of MM risk with OR estimates of 1.3–1.6 even after adjustment for nevus count, skin color, or freckle density. However, light eye color is correlated with other MM risk factor traits, including hair color, freckling, and light skin. Multivariate adjustments of eye color for these traits can result in loss of eye color as an independent predictor of MM risk (13). Therefore, eye color appears to be less powerful a predictor of MM than skin pigmentation, but it is also clear that that the relationship of pigmentation phenotypes with one another and with MM risk is complex. We propose that a better understanding of the genetic determinants of pigmentation phenotypes will help to tease apart these complex relationships and better elucidate the etiology of cutaneous neoplasms.

Our results suggest that the *P* gene may in part determine blue *versus* nonblue eye color, which is consistent with the predicted function of the putative *EYCL3* gene (1). It is possible that an unidentified locus in linkage disequilibrium with *P* gene is responsible for the effects observed here. However, the evidence from linkage analysis suggesting an eye color gene at this locus (1), and the known role of the *P* gene in determining predisposition to oculocutaneous albinism, provides support that this gene is responsible for the eye color effects reported here. These results indicate that the *P* gene, in part, determines normal phenotypic variation in human eye color.

Acknowledgments

We thank Dr. Robert D. Nicholls for helpful discussion of this research.

References

1. Eiberg, H., and Mohr, J. Assignment of genes coding for brown eye colour (BEY2) and brown hair colour (HCL3) on chromosome 15q. Eur. J. Hum. Genet., *4:* 237–241, 1996.

2. Korner, A., and Pawelek, J. Mammalian tyrosinase catalyses three reactions in the biosynthesis of melanin. Science (Wash. DC), *217:* 1163–1165, 1982.

3. Manga, P., Biossy, R. E., Pifo-Hirst, S., Zhou, B-K., and Orlow, S. J. Mislocalization of melanosomal proteins in melanocytes from mice with oculocutaneous albinism type 2. Exp. Eye Res., *72:* 695–710, 2001.

4. Brilliant, M. H. The mouse p (pink-eyed dilution) and human *P* genes, oculocutaneous albinism type 2 (OCA2), and melanosomal pH. Pigment Cell Res., *14:* 86–93, 2001.

5. Rinchik, E. M., Bultman, S. J., Hosthemke, B., Lee, S. T., Strunk, K. M., Spritz, R. A., Avidano, K. M., Jong, M. T., and Nicholls, R. D. A gene for the mouse pink-eye dilution locus and for human type II oculocutaneous albinism. Nature (Lond.), *361:* 72–76, 1993.

6. Lee, S. T., Nicholls, R. D., Bundey, S., Laxova, R., Musarella, M., and Spritz, R. A. Mutations of the *P* gene in oculocutaneous albinism, ocular albinism, and Prader-Willi syndrome plus albinism. N. Eng. J. Med., *330:* 529–534, 1994.

7. Spritz, R. A., Bailin, T., Nicholls, R. D., Lee, S. T., Park, S. K., Mascari, M. J., and Butler, M. G. Hypopigmentation in the Prader-Willi syndrome correlates with *P* gene deletion but not with haplotype of the hemizygous *P* allele. Am. J. Med. Genet., *71:* 57–62, 1997.

8. Walker, A. H., White, D. L., Jaffe, J. M., Kanetsky, P. A., and Rebbeck, T. R. Collection of genomic DNA by buccal swabs for PCR-based biomarker assays. Env. Health Persp., *107:* 3–7, 1999.

9. Kerr, R., Stevens, G., Manga, P., Salm, S., John, P., Haw, T., and Ramsay, M. Identification of *P* gene mutations in individuals with oculocutaneous albinism in sub-Saharan Africa. Hum. Mutat., *15:* 166–172, 2000.

10. Stevens, G., Ramsay, M., and Jenkins, T. Oculocutaneous albinism (OCA2) in sub-Saharan Africa: distribution of the common 2.7-kb *P* gene deletion mutation. Hum. Genet., *99:* 523–527, 1997.

11. Lee, S. T., Nicholls, R. D., Jong, M. T., Fukai, K., and Spritz, R. A. Organization and sequence of the human *P* gene and identification of a new family of transport proteins. Genomics, *26:* 354–363, 1995.

12. Bliss, J. M., Ford, D., Swerdlow, A. J., Armstrong, B. K., Cristofolini, M., Elwood, J. M., Green, A., Holly, E. A., Mack, T., MacKie, R. M., and The International Melanoma Analysis Group (IMAGE). Risk of cutaneous melanoma associated with pigmentation characteristics and freckling: systematic overview of 10 case-control studies. Int. J. Cancer, *62:* 367–376, 1995.

13. Holly, E. A., Aston, D. A., Cress, R. D., Ahn, D. K., and Kristiansen, J. J. Cutaneous melanoma in women. II. Phenotypic characteristics and other host-related factors. Am. J. Epidemiol., *141:* 934–942, 1995.

P Gene as an Inherited Biomarker of Human Eye Color

Timothy R. Rebbeck, Peter A. Kanetsky, Amy H. Walker, et al.

Cancer Epidemiol Biomarkers Prev 2002;11:782-784.