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Identification and functional characterization of natural human melanocortin 1 receptor (MC1R) mutant alleles in Pakistani population

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

SUPPLEMENTARY MATERIAL

Additional supporting information may be found in the online version of this article:

Methods S1. Families enrollment and functional studies.

Figure S1. Pakistani families segregating hypopigmentation phenotype.

Figure S2. *MC1R* mutations in Pakistani families.

Figure S3. Dose-response curves for stimulation of cAMP synthesis downstream of WT or mutant *MC1R*.

Figure S4. Competition radioligand binding analysis.

Table S1. Phenotype of probands of Pakistani families segregating hypopigmentation.

Table S2. SNP genotypes in 1,359,277 bp surrounding *MC1R* mutation.

Summary

Melanocortin 1 receptor (MC1R), a Gs protein-coupled receptor of the melanocyte's plasma membrane, is a major determinant of skin pigmentation and phototype. Upon activation by α -melanocyte stimulating hormone, MC1R triggers the cAMP cascade to stimulate eumelanogenesis. We used whole exome sequencing to identify causative alleles in Pakistani families with skin and hair hypopigmentation. Six *MC1R* mutations segregated with the phenotype in seven families, including a p.Val174del in-frame deletion and a p.Tyr298* nonsense mutation that were analyzed for function in heterologous HEK293 cells. p.Tyr298* MC1R showed no agonist-induced signaling to the cAMP or ERK pathways, nor detectable agonist binding. Conversely, signaling was comparable for p.Val174del and wild-type in HEK cells overexpressing the proteins, but binding analysis suggested impaired cell surface expression. Flow cytometry and confocal imaging studies revealed reduced plasma membrane expression of p.Val174del and p.Tyr298*. Therefore p.Tyr298* was a total loss-of-function (LOF) allele, while p.Val174del displayed a partial LOF attribute.

Keywords

MC1R; melanocortin 1 receptor; melanocytes; cAMP signaling; hypopigmentation

Skin pigmentation, one of the most visible phenotypes in humans, is a complex trait regulated by ~120 genes (Bennett and Lamoreux, 2003; Dessinioti et al., 2011). Proteins encoded by these genes subserve many functions that span the gamut from transcription factors (e.g. PAX3, SOX10, MITF) to receptors expressed at the plasma membrane of melanocytes (e.g. melanocortin 1 receptor, MC1R). In humans, epidermal melanocytes are the primary melanin-manufacturing units. Melanins are formed within melanosomes, which are transferred to keratinocytes and distributed throughout the skin (Yamaguchi et al., 2007).

Thirty large families (Figure 1, Figure S1) with hypopigmentation (Table S1) were recruited from different Pakistani regions after institutional review committees' approval (Methods S1). For genetic screening, we adopted whole exome enrichment followed by massive parallel sequencing. Bioinformatic analysis of sequencing data identified six *MC1R* variants co-segregating with the phenotype in seven of these families (Figure 1, Table 1). These variants were four missense substitutions, an in-frame deletion and a nonsense mutation (Table 1, Figure S2). The p.Asp294His allele found in family PKAB150 is a well documented 'red hair color' variant (R allele) in populations of European descent. Similarly, p.Gly89Arg, p.Ser172Ile and p.Val174del alleles have been reported (Landi et al., 2006; Pastorino et al., 2004), and the p.Ala171Gly variant appears in SNP public databases (Table 1). On the other hand, a c.894C>G (p.Tyr298*) variant which has not yet been reported to the best of our knowledge was found in one MC1R family (Table 1). SNPs linked to *MC1R* were genotyped in unrelated affected individuals of families PKAB168 and LUAB12, homozygous for the c.515G>T (p.(Ser172Ile)) mutation, and the flanking haplotype was consistent with a founder effect (Table S2). No mutations in other known pigmentation genes were found in these seven families, segregating *MC1R* alleles.

Affected individuals homozygous for *MC1R* alleles had reddish brown hair, pinkish white skin and light brownish irises, and skin freckles (Figure 1). No retinal degeneration, photophobia or nystagmus was observed. Additionally, a family history and physical and clinical evaluations of the two oldest affected individuals revealed no signs of hypotonia, obesity, impaired vestibular or cognitive abilities, seizures, hearing loss, cataracts or skin cancer.

Consistent with the phenotypes, all missense mutations were predicted damaging (Table 1). However, no reliable *in silico* predictions could be obtained for the p.Tyr298* mutation and the in-frame single amino acid deletion p.Val174del. MC1R contains 317 amino acids and the p.Tyr298* mutation should delete the cytoplasmic tail bearing the C-terminal pentapeptide (Figure S2B) required for normal cell surface expression (Sanchez-Mas et al., 2005). We performed functional studies of p.Tyr298* and p.Val174del in transiently transfected HEK293 cells. The electrophoretic pattern of the mutants and wild-type (WT) proteins were comparable, with bands corresponding to “*de novo*” and glycosylated MC1R monomers and dimers (Figure 2A). The intracellular stability of the proteins was assessed by treating transfected cells with 0.1 mM cycloheximide (CHX) for various times (Figure 2B) followed by plotting the intensity of residual signals as a semi-log graph (Figure 2C) for calculation of half-lives (3.9 h for WT, 2.1 h for p.Tyr298* and 1.6 h for p.Val174del). Therefore the p.Tyr298* and p.Val174del mutations significantly destabilized the receptor.

MC1R undergoes dimerization as an early biosynthetic step (Sanchez-Laorden et al., 2006). To verify if the mutants were able to heterodimerize with WT receptor, we performed co-immunoprecipitation studies using differentially epitope-labeled MC1R forms. Upon immunoprecipitation of WT, both p.Tyr298* and p.Val174del were detected in the immune pellets, indicating efficient heterodimerization (Figure 2D).

Next, we compared activation of cAMP signaling by WT and mutant receptors. Upon stimulation with NDP-MSH, cells expressing p.Val174del showed cAMP levels as high as WT. Conversely, p.Tyr298* was devoid of agonist-induced signaling to the cAMP pathway (Figure 3A). We also obtained dose-response curves for agonist concentrations from 10^{-11} to 10^{-6} M (Figure S3). Again, p.Tyr298* showed a complete loss-of-function (LOF), whereas dose-response curves were similar for p.Val174del and WT (EC_{50} ~10 and 30 nM, respectively). Agonist-stimulated MC1R activates the extracellular signal-regulated protein kinases ERK1 and ERK2 independently of cAMP (Herraiz et al., 2009; Herraiz et al., 2011). Accordingly, we checked the MC1R forms for ERK activation (Figure 3B). WT and p.Val174del comparably activated the ERKs upon stimulation with NDP-MSH, whereas the p.Tyr298* mutant was completely inactive.

To distinguish between inability of p.Tyr298* to bind agonist or, alternatively, failure to undergo an agonist-induced transition to the active state, we performed radioligand binding analysis using [125 I]NDP-MSH as tracer. p.Tyr298* did not show detectable binding. However p.Val174del displayed reduced albeit significant binding potential (Figure 3C and S4), with binding affinity comparable with WT (K_d 1.2 ± 0.3 and 3.4 ± 0.7 nM for p.Val174del and WT, respectively), but significantly lower B_{max} (11.7 ± 0.7 and 3.3 ± 0.2 femtomoles/ μ g protein for WT and p.Val174del, respectively). These data suggested that

p.Val174del was able to bind agonist with high affinity but was trafficked to the cell surface less efficiently than WT. Conversely, p.Tyr298* was unable to bind agonist, either because of complete intracellular retention or due to an aberrant conformation with very low affinity for the agonist.

We assessed the plasma membrane expression of the mutants by flow cytometry. We used expression constructs harboring the Flag epitope fused to the extracellular N-terminus, so as to detect specifically cell surface MC1R molecules in non-permeabilized cells. For both mutants, the staining intensity was comparable but lower than WT (Figure 3D), indicating intracellular retention of the variants. Nevertheless, the finding of a staining intensity above background and of residual binding for p.Val174del also suggested partial anterograde trafficking with significant plasma membrane expression. This was confirmed by confocal laser microscopy (Figure 3E). In this experiment, the misprocessed and fully retained p.Arg162Pro variant (Sanchez-Laorden et al., 2009) used as control was not stained in non-permeabilized cells. Finally, we wanted to confirm mis-trafficking of p.Val174del in melanocytic cells. HBL human melanoma cells were transfected with Flag-labeled WT, p.Val174del or p.Tyr298* as a control. Then, cells were stained with a phycoerythrin-labeled anti-Flag monoclonal and analyzed in a flow cytometer (Figure 3F). In keeping with the results obtained in heterologous HEK cells, both variants showed a comparable staining intensity, markedly lower than WT. We also reacted non-permeabilized HBL cells expressing WT or p.Val174del with a peroxidase-labeled anti-Flag antibody, followed by detection of cell surface receptor using a peroxidase substrate (Figure 3G). This procedure again confirmed reduced cell surface expression of p.Val174del expressed in melanocytic cells.

Overall, our functional analysis showed that p.Tyr298* was a total LOF mutant, significantly mistrafficked and unable to bind agonists. This confirmed previous findings on the importance of the cytosolic extension for normal trafficking (Mas et al., 2003), but shows that residual movement to the cell surface remains possible even in the absence of this major structural determinant. On the other hand, p.Val174del efficiently activated the cAMP and the ERK pathways, but its cell surface expression was reduced according to flow cytometry, confocal microscopy, and radioligand binding experiments. This impaired anterograde trafficking did not result in decreased signaling parameters in a heterologous overexpression system where exogenous MC1R should be in excess over downstream components of the signal transduction machinery (Mas et al., 2003). However, in normal melanocytes expressing hundreds or a few thousands receptors per cell, MC1R should be the limiting factor. In these physiological conditions, any reduction in cell surface expression should cause a similar decrease in the intracellular response (Mas et al., 2003). In summary, consistent with the observed pigmentation phenotypes, our functional data allow predicting that p.Tyr298* MC1R should behave as a strong R-type RHC allele, while the p.Val174del mutant might display a partial LOF. Our results would be useful for future diagnosis, genetic counseling, molecular epidemiology and functional studies of MC1R-associated skin tone disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

Here, we documented *MC1R* alleles in Pakistani families segregating skin hypopigmentation. Our data provide further insight on the relationship of *MC1R* status with human pigmentation and support the current view that functional characterization of new variants might allow for a prediction of phenotypic effects for low frequency alleles that cannot be reliably analyzed by genetic epidemiology association studies, or for mutations not easy to analyze *in silico*. Our results would be useful for future diagnosis, genetic counseling, molecular epidemiology and functional studies of *MC1R*-associated skin tone disorders. They also reveal the complexity of determinants of normal *MC1R* trafficking to the plasma membrane.

Figure 1. Hypopigmentation phenotypes in seven Pakistani families co-segregates with MC1R mutations

(A) Filled and empty symbols represent affected and unaffected individuals, respectively. A double line uniting two individuals represents consanguineous marriage. Arrows indicate the affected individuals screened via whole exome sequencing. Genotypes of all the participating individuals are also shown. (B) Photographs of individuals homozygous (A) for *MC1R* alleles. Some of the probands have used hair dyes. For comparison of skin tone, normal (N) individuals from *MC1R* families are also shown.

Figure 2. Expression and stability of p.Tyr298* and p.Val174del MC1R

(A) HEK cells transfected with Flag epitope-labelled MC1R forms were detergent-solubilized. Aliquots were Western-blotted with an anti-Flag antibody. Comparable loading was ascertained by staining for ERK2 and GAPDH (lower blot). The mobility of p.Tyr298* was slightly higher, consistent with deletion of 19 C-terminal amino acids. (B) Intracellular stability of MC1R variants. Cells expressing the indicated MC1R forms were treated with cycloheximide (CHX) from 30 min to 7.5 hours. Residual levels of MC1R were estimated by Western blot. (C) Cells transfected to express WT or mutant MC1R were treated with 0.1

mM cycloheximide (CHX) for times from 0.5 to 7.5 h, washed and detergent-solubilized. Aliquots were electrophoresed and blotted with anti-Flag. The intensity of receptor bands in the blots was quantitated with ImageJ. The residual signals were plotted as a semi-log graph for calculation of half-lives. **(D)** Co-immunoprecipitation of WT and variant MC1R. HEK cells were transfected with WT MC1R bearing a C-terminal HA tag, alone or in combination with Flag-labeled WT, p.Tyr298* or p.Val174del. Extracts were immunoprecipitated with anti-HA, and the immune complexes were analyzed by Western blot using an anti-Flag. A blot representative of 3 experiments is shown.

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Figure 3. Functional analysis of p.Tyr298* and p.Val174del MC1R

(A) cAMP levels in cells expressing the mutants after stimulation with saturating NDP-MSH (10^{-6} M, 30 min). cAMP was estimated by an enzyme immunoassay (results given as mean $\pm$ SEM, $n = 6$, p values calculated with GraphPad software. ns, not significant). (B) Functional coupling of p.Tyr298* and p.Val174del to the ERKs. PC12 cells expressing the MC1R forms were stimulated with NDP-MSH (10^{-6} M) for the times shown, and solubilized in the presence of phosphatase inhibitors. Aliquots were electrophoresed, blotted and probed for phosphorylated ERKs (pERK1/2, upper blot). Membranes were reprobed for

ERK2 to ascertain comparable loading (middle) and for MC1R to assess transfection (lower blot). (C) Equilibrium binding of [125 I]NDP-MSH. Transfected cells were incubated with 10^{-9} M [125 I]NDP-MSH for 1 h, washed, and the radioactivity associated was determined (mean $\pm$ SEM, n = 6). (D) Flow cytometric analysis of cell surface expression. Non-permeabilized cells expressing the indicated MC1R forms were stained and 10,000 events were analysed by flow cytometry. The filled curve is a blank obtained with cells transfected with empty vector. Histograms corresponding to WT and variant MC1R are shown in red for p.Tyr298* (p.Y298*), blue for p.Val174del (p.V174del) and black for WT. Representative histograms out of triplicates are shown. (E) Confocal microscopy demonstration of receptor variants on the cell surface of HEK293 cells. Cells grown on coverslips were transfected as indicated and, if required, permeabilized with 0.5% Igepal, 15 min at 4°C. Cells were stained for MC1R (green) with anti-Flag followed by an Alexa 488-conjugated secondary antibody. Nuclei were stained with 7-AAD (red). The misprocessed and fully retained p.Arg162Pro variant (Sanchez-Laorden et al., 2009) used as control was not stained in non-permeabilized cells. Bar size: 42 μ M. (F) Flow cytometric analysis of cell surface expression of WT and variant MC1R expressed in HBL human melanoma cells. Transfection, analysis and graph as in panel D. (G) Comparison by an ELISA procedure of cell surface expression of WT and p.Val174del expressed in human HBL melanoma cells.

Table 1*MC1R* variants and their predicted effect on the encoded protein

Family ID	Pathogenic variants*	Polyphen2	SIFT	Mutation Taster	Allele frequency	1000 Genome Browser	NHLBI exome variants	Reference
PKAB161	c.265G>C (p.(Gly89Arg))	Possibly Damaging	Damaging	Polymorphism	0/186	0.000	1/12943	(McKenzie et al., 2003)
PKAB181	c.512C>G (p.(Ala171Gly))	Benign	Damaging	Disease causing	0/186	--	2/12970	dbSNP141 database rs376670171
PKAB168, LUAB12	c.515G>T (p.(Ser172Ile))	Probably Damaging	Damaging	Polymorphism	0/186	0.001	1/12969	(Landi et al., 2006)
PKAB169	c.520_523delGTC (p.(Val174del))	--	--	Disease causing	0/186	--	--	(Pastorino et al., 2004)
PKAB150	c.880G>C (p.(Asp294His))	Probably Damaging	Damaging	Disease causing	0/186	0.003	205/12519	(Valverde et al., 1995)
PKAB156	c.894C>G (p.(Tyr298*))	--	--		0/186	--	2/12666	In this study