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Functional assessment of tyrosinase variants identified in individuals with albinism is essential for unequivocal determination of genotype-to-phenotype correlation

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Summary

Background Oculocutaneous albinism type 1 (OCA1), caused by pathogenic variations in the tyrosinase gene (*TYR*), is the most frequent and severe form of hypopigmentary disorder worldwide. While OCA1A manifests as a complete loss of melanin pigment, patients with OCA1B show residual pigmentation of the skin, hair and eyes. Limited experimental evidence suggests retention of TYR in the endoplasmic reticulum (ER) causes OCA1 pathogenesis. However, a comprehensive functional analysis of TYR missense variations and correlation with genotype is lacking.

Objectives Functional characterization of nonsynonymous tyrosinase variants in patients with OCA1 reported in the Albinism Database, dbSNP and the published literature, and an attempt to correlate them with reported and predicted phenotypes.

Methods Thirty-four reported missense variants of TYR were subcloned by site-directed mutagenesis, and the dual-enzyme activities of the variant proteins were compared with the wild-type. The degree of ER retention was also checked for each of the variants through endoglycosidase H (Endo H) digestion followed by immunoprecipitation and densitometric analysis.

Results Functional studies revealed one reported OCA1A variation with nearly 100% enzyme activity, 10 OCA1B variants lacking any enzyme activity, eight nonsynonymous single-nucleotide polymorphisms (SNPs) with ~30–70% of enzyme activity, and three SNPs that completely lacked activity altogether. The Endo H assay corroborated these results.

Conclusions Loss of enzyme activity of TYR variants was completely in agreement with ER retention across all variants examined. The results of the assay clearly established that determination of the biological activity of identified variants in patients with OCA is essential to correlate the identified suspect genotype with the obvious phenotype of the disease.

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Conflicts of interest

None declared.

M.M and M.S. contributed equally to this study.

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What's already known about this topic?

- Oculocutaneous albinism (OCA) is a group of autosomal recessive disorders resulting from congenital hypopigmentation of ocular and cutaneous tissues.
- Defects in the tyrosinase gene (*TYR*) are a major cause of OCA in many countries. Incidentally, OCA is a major cause of childhood blindness in India.
- Limited experimental evidence suggests that retention of TYR in the endoplasmic reticulum (ER) is responsible for OCA1 pathogenesis.

What does this study add?

- This study reveals that ER retention of tyrosinase variant proteins is the principal cause of loss of enzyme activity.

- The study clearly demonstrates that a functional assay of suspect TYR variants is essential to correlate genotype to phenotype in patients with OCA.
- *TYR* single-nucleotide polymorphisms listed in dbSNP, specifically with low or no recorded frequency, could potentially be mutant alleles identified in phenotypically normal heterozygous individuals.

What is the translational message?

- Using OCA1 as a model disease, it is proposed that identification of rare or unique genetic variants in the causal gene of an inherited disease must be complemented with functional studies to implicate the suspect variants with the disease unequivocally.
- For genetic diseases, correct determination of causal mutations has far-reaching implications for genetic counselling and/or prenatal diagnosis, as appropriate.

Tyrosinase (TYR), the rate-limiting enzyme in the melanin biosynthetic pathway, has been implicated in regulating the variation of normal pigmentation, in addition to its role in precipitating the hypopigmentary disease oculocutaneous albinism type 1 (OCA1).[1] Some patients with OCA1 present with residual pigmentation and are classified as having OCA1B,[2,3] in contrast to patients with OCA1A, who are completely devoid of melanin.[4,5]

Tyrosinase, a type I membrane-bound melanosomal glycoprotein, requires a long processing time for maturation, most of which is required for protein folding. The whole process of sorting, folding, targeting and intracellular trafficking of TYR takes place in an extremely concerted fashion.[6] TYR is transported cotranslationally to the endoplasmic reticulum (ER), mediated by an N-terminal signal peptide. There the 60-kDa core polypeptide is modified by the addition of multiple 2·5-kDa, flexible, hydrophilic, N-linked glycans, producing the 70-kDa species. These N-linked glycans are trimmed by resident glucosidases (glucosidases I and II), and the resulting monoglucosylated oligomannosidic glycans interact with chaperone molecules, namely membrane-bound calnexin and soluble calreticulin (the paralogue of calnexin), for correct folding.

At this stage, the TYR species is sensitive to endoglycosidase H (Endo H), which cleaves asparagine-linked mannose-rich oligosaccharides added in the ER, but not the complex oligosaccharides from glycoproteins that are added later in the Golgi apparatus. In the Golgi apparatus, the oligomannosidic N-linked glycans are processed to complex glycans, which further increase the molecular mass of TYR, resulting in an 80-kDa protein resistant to Endo H digestion. In the trans-Golgi network, two copper ions are loaded onto TYR; however, recent studies show that such copper loading is a transient phenomenon, and copper is reloaded in the melanosomes.[7,8] It can be assumed that pathogenic variations in TYR could hamper any of the above-mentioned processes, leading to defects in melanin biosynthesis and thus OCA.

In the Albinism Database (http://www.ifpcs.org/albinism/index.html; maintained by the University of Minnesota, and updated until September 2009), 234 TYR variants have been reported as pathogenic, of which 163 are missense changes. Interestingly, six different *in vitro* functional studies[9–14] have reported complete ER retention of 13 missense TYR pathogenic variations, and based on this, OCA1 has been suggested to be an ER retention disorder. It is well known that misfolding or aggregation of proteins leads to their retention in the ER, and ER-associated degradation. ER quality-control function ensures the sorting of only correctly processed proteins for transport to the Golgi apparatus. However, all residues do not play an equivalent role in protein folding. Therefore, not all variants would be expected to lead to ER-retained protein. This raises a potential question of whether ER retention could actually be the universal cause of OCA1 pathogenesis, considering the huge number of missense mutations that are yet to be characterized.

Twenty-five TYR variants have been associated with OCA1B on the basis of clinical features. However, the molecular mechanism of causation of OCA1B is not yet fully understood. We thought it would be interesting to examine whether the variants associated with OCA1B lead to partial retention of TYR variants, leading to residual enzyme activity, as opposed to complete ER retention and null activity for cases of OCA1A.

Recently, it has been hypothesized that the clinical variability of OCA depends on the background pigmentation of the affected individual. Thus, an individual with a darker complexion would be expected to require two pathogenic variations to precipitate the disease, owing to a high pigmentation threshold. On the other hand, in an individual with a lighter complexion, a single severe pathogenic variation along with a hypomorphic variant (which could be a single-nucleotide polymorphism, SNP) might knock down the normal pigmentation pathway, causing the disease.[15] This hypothesis brings forth a new angle for studying the biological function of the nonsynonymous SNPs of *TYR* with respect to disease pathogenesis, as well as the regulation of normal pigmentation variation.

In this study, we wanted to assess the enzyme activity and ER retention of hitherto uncharacterized TYR missense variants

in different domains of the protein. This would help us to understand (i) whether ER retention is the principal cause of OCA1 pathogenesis, (ii) the molecular basis of OCA1B and (iii) the potential implications of TYR nonsynonymous SNPs towards disease pathogenesis and normal pigmentation variation. To address these issues we characterized eight TYR variants associated with OCA1A, 14 variants associated with OCA1B and 12 nonsynonymous SNPs reported in the Albinism Database, dbSNP (http://www.ncbi.nlm.nih.gov/SNP) and the published literature.[16] We selected 34 variants spanning the entire length of the protein, such that all predicted domains of TYR are represented in our study panel.

Materials and methods

Cell line and reagents

Immortalized human embryonic kidney (HEK293) cells were used for transfection of wild-type (WT) and variant TYRs. HEK cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) at pH 7·4 supplemented with 10% fetal bovine serum (FBS), containing 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 0·25 μg mL^{-1} amphotericin B. All of these reagents were procured from Thermo Fisher Scientific Inc. (Waltham, MA, U.S.A.).

Site-directed mutagenesis, construction of expression vectors and transfection

The pEGFP-TYR-WT vector (WT human TYR vector tagged with enhanced green fluorescent protein, with Ser at position 192) used in the study was kindly donated by Ruth Halaban from Yale University School of Medicine, New Haven, CT, U.S.A. Eight OCA1A variants, 14 variants associated with OCA1B and 12 nonsynonymous SNPs of TYR (Fig. 1) were individually inserted into the pEGFP-TYR-WT vector through site-directed mutagenesis (QuikChange XL Site-Directed Mutagenesis Kit; Agilent, Santa Clara, CA, U.S.A.).

Transient transfection in HEK293 with WT TYR, as well as TYR variants, was done with Lipofectamine 2000 according to the manufacturer's protocol (Thermo Fisher Scientific Inc.). Briefly, cells were grown to 75–80% confluence in a 35-mm transfection dish, and 2 μg of each expression vector was mixed with 3 μL of Lipofectamine 2000 solution (stock 1 mg mL^{-1}) in serum-free DMEM. HEK293 cells were incubated with the transfection mixture for 5 h, the media was discarded followed by incubation in fresh DMEM containing 10% FBS in 5% CO_2 at 37 °C for 48 h, and the cells were harvested. β-Galactosidase was cotransfected with TYR in HEK293 cells to assess the efficiency of transfection.

Preparation of whole-cell lysate

For functional analysis of TYR, proteins were extracted from cells by sonication in lysis buffer (20 mmol L^{-1} Tris, pH 7·4, 150 mmol L^{-1} NaCl, 2 mmol L^{-1} ethylenediaminetetraacetic acid, 1% NP-40 detergent, 1% protease inhibitor cocktail). The supernatants were collected and used for enzyme assays, and the protein contents estimated by standard Bradford assay (BioRad, Hemel Hempstead, U.K.).

Tyrosinase activity

Both the tyrosine hydroxylase and dopa oxidase activities of TYR were evaluated independently for each of the variant and WT enzymes.

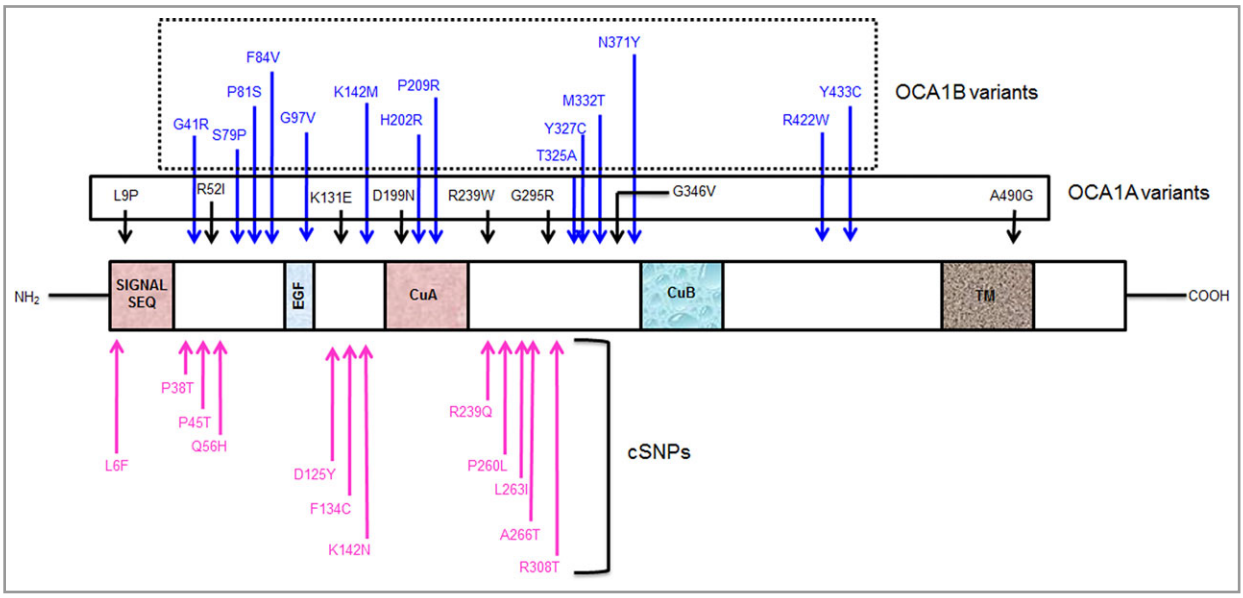

Fig 1. Distribution of 34 tyrosinase variants selected in our study across the protein. TM, transmembrane domain; CuA and CuB: copper A and B domains, respectively; EGF, epidermal growth factor; cSNP, coding single-nucleotide polymorphism; OCA, oculocutaneous albinism.

Analysis of tyrosine hydroxylase activity

Tyrosine hydroxylase activity was assayed according to the method of Pomerantz.[17] In each well of the microtitre plate, 50 μL of reaction volume was prepared by mixing 25 μg total protein with sodium phosphate buffer (pH 7·2), bovine serum albumin, ^{3}H-tyrosine and L-dopa (as the cofactor and the proton donor). The assay was performed by incubating the mixture at 37 °C for 1 h, then transferring the contents in Eppendorf tubes containing a column with Celite 545 and Norit A in 950 μL of 0·1 mol L^{-1} HCl, followed by 1-h incubation at 23 °C. After centrifuging the tubes, 200 μL (20% of the total) of the supernatant was used for the determination of radioactivity by measuring the release of tritiated water ($^{3}H_2O$) from ^{3}H-tyrosine using cocktail-W in a liquid scintillation counter (LKB Wallac 1209 Rackbeta; Wallac Oy, Turku, Finland). The data were normalized with respect to β-galactosidase activity per microgram protein using the β-galactosidase activity assay kit from Promega Co. (Madison, WI, U.S.A.).

Analysis of dopa oxidase activity

Dopa oxidase activity was assayed spectrophotometrically according to the method of Nakazawa *et al.*,[18] using L-dopa as the substrate. Oxidation of L-dopa converts it to dopa quinone, which could be estimated by an enzyme-linked immunosorbent assay (ELISA) reader (EMax Precision Microplate Reader; Molecular Devices, Downingtown, PA, U.S.A.). A 20-μg sample of protein was incubated in 200 μL 0·5 mmol L^{-1} sodium phosphate buffer (pH 7·2) containing 0·1% L-dopa (in triplicate) for 1 h at 37 °C. The absorbance (measure of the dopa oxidase activity) was then monitored at 450 nm in an ELISA reader and compared with purified mushroom TYR (Sigma Chemical Co., St Louis, MO, U.S.A.) as standard. The data were normalized with respect to β-galactosidase activity per microgram protein using the β-galactosidase activity assay kit from Promega Co. The β-galactosidase activities for each of the transfection experiments were found to range between the mean ± 3 SD as measured by Microsoft Excel statistics, thus signifying that transfection efficiency was similar in each case.

Western blotting and endoglycosidase H digestion

Cell lysates (50 μg of total protein per well) were separated in 10% sodium dodecylsulfate polyacrylamide gels and transferred electrophoretically to polyvinylidene difluoride membrane (0·45 μm; Millipore Corporation, Bedford, MA, U.S.A.). TYR was detected with primary antibody, antihuman TYR (H-109) (1 : 100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), and secondary antibody, antirabbit, conjugated with alkaline phosphatase (Santa Cruz Biotechnology) at 1 : 1000 dilution. The cell extracts (60 μg of total protein) were digested with 1000 U of Endo H (New England Biolabs, Beverly, MA, U.S.A.) for 3 h at 37 °C. After the digestion, the cell extracts were mixed with Tris-glycine-SDS sample buffer supplemented with 1% 2-mercaptoethanol and boiled for 5 min. Samples were subjected to SDS polyacrylamide gel electrophoresis, and immunoreactive bands were detected by Western blotting using the TYR (H-109) antibody. The Endo H-resistant and -sensitive bands were quantified using ImageJ software (http://imagej.net), and the optical densities were calculated from calibrated optical density step tables from ImageJ (Table S1).

SIFT and PolyPhen-2 analysis

We analysed all of the selected variants through SIFT (http://sift.jcvi.org) and the PolyPhen-2 HumVar model (http://genetics.bwh.harvard.edu/pph2), two commonly used *in silico* predictors of the deleterious effects of missense variants. SIFT uses a sequence-homology-based approach to classify the substitutions into tolerant and intolerant (cut-off score < 0·05). PolyPhen-2 predicts the possible impact of an amino acid substitution on the structure and function of a human protein based on a number of features, including the sequence and phylogenetic and structural information characterizing the substitution. Variants with increasing deleterious nature are appraised as benign, possibly damaging or probably damaging.

Correlation coefficient assessment

Correlation between the ratio of the optical density of the Endo H-resistant (lower-mobility upper band) and Endo H-sensitive bands (higher-mobility lower band) (Fig. 2) and the tyrosine hydroxylase and dopa oxidase activities of TYR variants were calculated using Microsoft Excel statistics.

Results

Analysis of tyrosine hydroxylase and dopa oxidase activities

Five of eight OCA1A variants were found to be devoid of any enzyme activity, namely p.L9P, p.R52I, p.K131E, p.R239W and p.G295R (Fig. 2). However, two OCA1A variants (p.G346V and p.D199N) retained residual enzymatic activities, while p.A490G showed activity similar to the WT enzyme (Fig. 2).

Unlike with cases of OCA1A, true OCA1B variants would be expected to have partial enzyme activity, leading to the residual melanization found in patients with OCA1B. Surprisingly, 10 of the 14 tested variants did not retain any activity (p.P81S, p.F84V, p.G97V, p.K142M, p.H202R, p.P209R, p.Y327C, p.M332T, p.N371Y and p.R422W), while only two (p.G41R and p.Y433C) showed residual activity. The two remaining variants (p.S79P and p.T325A) showed activity similar to the WT enzyme (Fig. 2). The enzyme activities of all of the above-mentioned variants are shown in Table 1.

Similar experiments with the selected coding SNPs revealed eight of the 12 variants to be potentially functional, retaining ~30–70% of WT enzyme activity (Fig. 2). Most surprisingly,

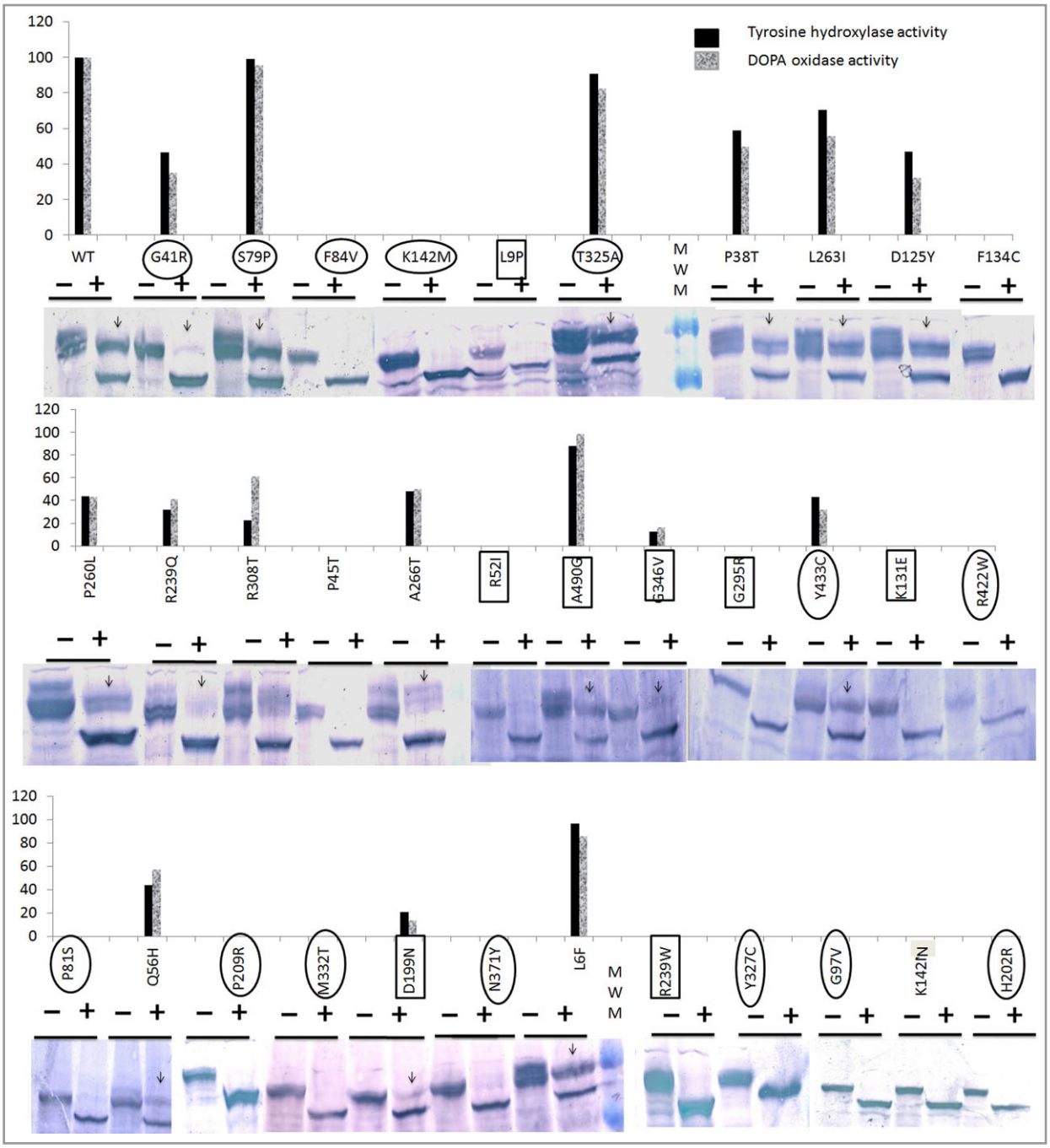

Fig 2. Assessment of the biological activity of the tyrosinase variants and their processing in the endoplasmic reticulum (ER). Tyrosine hydroxylase and dopa oxidase activities of selected variants are shown as a bar diagram in the upper panel, and the endoglycosidase H (Endo H) sensitivity of the variant proteins by polyacrylamide gel electrophoresis bands in the lower panel (+, Endo H treated; −, untreated) in each of the three sets. Endo H-treated (+) variants that retained enzyme activity showed a distinct Endo H-resistant form, represented by the lower-mobility upper band (marked with an arrow) along with an Endo H-sensitive higher-mobility lower band. Oculocutaneous albinism (OCA)1A variants are shown in rectangular boxes, OCA1B variants are encircled in oval shapes and single-nucleotide polymorphisms (SNPs) are shown without any boundary. Contrary to expectation, two reported OCA1A variants (D199N and G346V) were found to have some residual activities, whereas A490G showed activity similar to the wild-type. Two reported OCA1B variants (G41R and Y433C) showed residual activities as expected. However, two other variants (S79P and T325A) showed enzyme activities similar to the wild-type, and as many as 10 variants were found to retain no enzyme activity. Nine of 12 reported SNPs showed a wide variation in enzyme activities relative to the control. However, three SNPs (P45T, F134C, K142N) completely lacked any enzyme activity, suggesting that these variants belong to the OCA1A type. The ER retention data are completely consistent with the enzyme activities of the variants.

Table 1 Assessment of tyrosinase activities in the variants reported in cases of oculocutaneous albinism (OCA) types 1A and 1B

Selected variant		TH activity		DO activity			
Nucleotide change	Amino acid change	U mg^{-1}	% of WT	U mg^{-1}	% of WT	SIFT prediction	PolyPhen-2 prediction
WT	WT	501·1	100	1192·3	100	–	–
OCA1A variants							
c.26T>C	p.L9P	0	0	0	0	Damaging	Probably damaging
c.155G>T	p.R52I	0	0	0	0	Damaging	Probably damaging
c.391A>G	p.K131E	0	0	0	0	Damaging	Probably damaging
c.595G>A	p.D199N	103·5	20·7	158·9	13·3	Damaging	Probably damaging
c.715C>T	p.R239W	0	0	0	0	Damaging	Probably damaging
c.883G>A	p.G295R	0	0	0	0	Damaging	Probably damaging
c.1037G>T	p.G346V	62·1	12·4	195·8	16·4	Damaging	Probably damaging
c.1469C>G	p.A490G	441·5	88·1	1175	98·6	Tolerated	Benign
OCA1B variants							
c.121G>A	p.G41R	232·8	46·5	416·9	34·9	Damaging	Probably damaging
c.235T>C	p.S79P	496	99·01	1139·3	95·6	Tolerated	Possibly damaging
c.241C>T	p.P81S	0	0	0	0	Damaging	Probably damaging
c.250T>G	p.F84V	0	0	0	0	Damaging	Probably damaging
c.290G>T	p.G97V	0	0	0	0	Damaging	Probably damaging
c.425A>T	p.K142M	0	0	0	0	Damaging	Probably damaging
c.605A>G	p.H202R	0	0	0	0	Damaging	Probably damaging
c.626C>G	p.P209R	0	0	0	0	Damaging	Probably damaging
c.973A>G	p.T325A	454·3	90·7	980·6	82·3	Tolerated	Possibly damaging
c.980A>G	p.Y327C	0	0	0	0	Damaging	Probably damaging
c.995T>C	p.M332T	0	0	0	0	Damaging	Probably damaging
c.1111A>T	p.N371Y	0	0	0	0	Damaging	Probably damaging
c.1264C>T	p.R422W	0	0	0	0	Damaging	Probably damaging
c.1298A>G	p.Y433C	223·1	44·5	383·6	32·2	Damaging	Probably damaging

TH, tyrosine hydroxylase; DO, dopa oxidase; WT, wild-type.

three SNPs (p.F134C, p.P45T and p.K142N) had no enzyme activity, while a single reported SNP (p.L6F) showed activity similar to the WT (Fig. 2; Table 2). For most SNPs, data for heterozygosity and minor allele frequency are either not available or are too low (Table 2) to consider them to be truly neutral SNPs, as also suggested by our experimental evidence.

Assessment of ER retention of the variant tyrosinase proteins by endoglycosidase H assay

In the Endo H sensitivity study with WT TYR, the lower-mobility band corresponds to mature TYR (hence Endo H resistant), and the higher-mobility band (Endo H-sensitive glycoform) represents incompletely modified protein in the ER. Five OCA1A variants that lacked any enzyme activity were found to be completely stuck in the ER, as evident from the absence of Endo H-resistant bands. Variant p.A490G, which retained almost WT-like activity, was also found to have an Endo H-resistant glycoform similar to the WT protein, while p.G346V and p.D199N were shown to retain partial activity, represented by very little release of the matured glycoform from the ER even after Endo H treatment (Fig. 2).

Similarly, the OCA1B variants and the coding SNPs with null activity were found to be completely ER retained, showing no Endo H-resistant band, while those having residual or almost WT-like enzyme activities resulted in proportional release of the matured TYR glycoform from the ER (Fig. 2). Densitometric analysis of the ratio of Endo-H resistant and Endo H-sensitive bands revealed that the amount of the ER-released fraction could be correlated to the enzyme activities ($r = -0{\cdot}75$ and $r = -0{\cdot}74$ for tyrosine hydroxylase and dopa oxidase, respectively) (Table S1).

Discussion

Oculocutaneous albinism type 1 has been suggested to be an ER retention disorder, where the variant TYRs remain trapped in the ER until they are degraded by the ER-associated degradation system.[9] This hypothesis has been proposed based on functional characterization of 13 missense variations reported in the Albinism Database. However, if ER retention is the sole cause of OCA1, it would be expected that variations that cause OCA1B would have partial enzyme activity and ER retention as well; this correlation has not been examined. We also thought it would be interesting to ascertain whether loss of activity of TYR, and ER retention of the TYR variant protein are context dependent – contingent on the location of the residue.

Our cell-based assays with reported OCA1A and OCA1B variations, as well as nonsynonymous SNPs located across the different domains of the protein, indeed suggested ER retention to be universally associated with the loss of enzyme

Table 2 Assessment of tyrosinase enzyme activities for variants reported as coding single-nucleotide polymorphisms (cSNPs) in the dbSNP database

Nonsynonymous cSNPs			Homozygous for minor allele (if any)	Heterozygosity	Tyrosine hydroxylase activity		Dopa oxidase activity		SIFT prediction	Polyphen-2 prediction
dbSNP ID	Nucleotide change	AA change			U mg^{-1}	% of WT	U mg^{-1}	% of WT		
	WT	WT	–	–	501·1	100	1192·3	100	–	–
rs76180653	c.18G>T	p.L6F	No	ND	563·9	112·6	1079·0	90·5	Tolerated	Benign
rs61759520	c.112C>A	p.P38T	No	ND	295·7	59·02	593·7	49·8	Tolerated	Benign
rs13312739	c.133C>A	p.P45T	No	ND	0	0	0	0	Tolerated	Probably damaging
rs11545464	c.168G>T	p.Q56H	No	ND	253·3	50·5	715·8	60·03	Damaging	Probably damaging
rs13312741	c.373G>T	p.D125Y	No	0·012	234·9	46·9	383·0	32·12	Damaging	Possibly damaging
rs33955261	c.401T>G	p.F134C	No	0·012	0	0	0	0	Damaging	Probably damaging
rs11545463	c.426G>T	p.K142N	No	0·002	0	0	0	0	Damaging	Probably damaging
rs36006590	c.716G>A	p.R239Q	No	0·001	186·1	37·1	519·0	43·5	Damaging	Probably damaging
rs11826502	c.779C>T	p.P260L	Yes	0·018	220·3	43·9	538·9	45·2	Damaging	Probably damaging
rs61759522	c.787C>A	p.L263I	No	ND	352·6	70·4	663·4	55·6	Tolerated	Benign
rs34297847	c.796G>A	p.A266T	No	ND	281·5	56·2	624·9	52·4	Tolerated	Probably damaging
rs1042608	c.923G>C	p.R308T	No	ND	130·6	26·06	765·1	64·2	Tolerated	Possibly damaging

AA, amino acid; WT, wild-type; ND, not determined.

activity, and the fraction of the enzyme released from the ER could be correlated with the residual enzyme activity. The results indicate that for the variants with null activity, the protein was completely retained in the ER, whereas those showing residual activity were only partially retained in the ER.

Our functional assays revealed some interesting observations: (i) three reported causal variants were found to represent innocuous variants; (ii) on the contrary, three reported coding SNPs showed complete null activity, thus representing OCA1A pathogenic variations; and (iii) 10 of the 14 variants identified in cases of OCA1B were null for enzyme activity, contrary to what is expected of true 'OCA1B' pathogenic changes. Of the reported causal variations for OCA1A that revealed residual enzyme activity, it was found in the original publication that the p.D199N variant was reported together with p.H180N and p.E398V.[19] It is possible that in the patient concerned, p.H180N and p.E398V – and not p.D199N – were the true causal variants leading to OCA1A.

Among the above-mentioned cases where the characterized variant did not retain any enzyme activity, we found that for four patients with OCA1B, the causal variation in the second allele could not be identified. The unidentified causal variations could potentially lead to leaky activity of TYR, and thus the OCA1B phenotype. But in five of the six remaining cases, the reported genotypes at the *TYR* locus do not support the phenotype described (Table 3). This raises a question of the 'pathogenic variation' (1A and 1B types) and 'SNP' status of the TYR variants in the various public-domain databases. This study demonstrates that genotype–phenotype correlation should always be based on functional evaluation of the underlying suspect genetic variants to avoid erroneous conclusions.

A few possibilities could explain the cases where the phenotype of the patient did not match our molecular assessment. Firstly, the clinical diagnosis is correct but there is actually a problem in segregation, and both of the identified mutations assessed lie in the same chromosome, while the other mutation remained uncharacterized; secondly, in the case of OCA1A, where the assessed mutant – harbouring residual enzyme activity – is not the actual causal variant but is in phase with the other uncharacterized variant; and thirdly, in the case of OCA1B, as appearance of the phenotype is subtle in terms of background pigmentation level and age, there could be an error in clinical diagnosis, although this possibility is less likely to occur. Our study highlights that if there is a lack of resources and the parental DNA is unavailable, molecular diagnosis could help delineate the true mutations causal to the disease.

Pigmentation is a quantitative trait regulated by polymorphic variants (SNPs) in genes associated with melanin synthesis and transport. At least 11 genes are known to be associated with normal pigmentation variation,[20] of which TYR is a major contributor through its Ser192Tyr SNP (rs1042602).[13,21] Eight of the 12 coding SNPs selected for functional validation retained enzyme activity in the range of ~30–70%. The clinical spectrum of OCA has been predicted to depend upon the pigmentation threshold of an affected individual and, thereby, the ethnicity. In individuals with a lineage of lighter complexion, a single severe variation plus a hypomorphic allele (SNP) can knock down the normal pigmentation pathway.[15] The SNPs with ~30–70% enzyme activity could thus represent hypomorphic alleles, which, when complemented with a deleterious variation in *TYR*, could tip the balance towards a pathogenic outcome and lead to OCA in population groups with a very fair skin type (i.e. low melanin background).

Our attempt to correlate the enzyme activity data and the *in silico* pathogenic variation prediction tools' data for individual

Table 3 Functional characterization of tyrosinase (TYR) variants reported in cases of oculocutaneous albinism (OCA) type 1B, and evaluation of genotype–phenotype correlation

Study	Selected variant; OCA type	TH/DO activities (% of WT)	Level of ER retention	Second variation	Was segregation analysis done?	Comments	Possible explanation
King 2003[23]	p.G41R; OCA1B	46·5/34·9	Partial	IVS2+2T>G; IVS2−7T>A	Yes	p.G41R and IVS2+2T>G in same allele; G41R has been assigned SNP ID rs369291837	If IVS2+2T>G causes splice variation, effect of p.G41R might be irrelevant. Better understanding depends on assessment of potential splice variation by the changes in IVS2 in both alleles
King 2003[23]	p.S79P; OCA1B	99·0/95·6	Similar to WT	UCM	Yes	–	The causal variations in TYR might not have been identified or the underlying genetic predisposition might be due to another OCA locus and not OCA1
King 2003[23]	p.P81S; OCA1B	0/0	Total	p.R402X	Yes	p.R402X is a nonsense change that is likely to inflict a severe effect	Functional characterization suggests OCA1A phenotype, and is not consistent with OCA1B as reported in the Albinism Database
Hutton 2008[24]	p.F84V; OCA1B	0/0	Total	UCM	Not reported	Presence of the temperature-sensitive variant p.R402Q (rs1126809) (hom), the hypomorphic variant p.S192Y (rs1042602) (het) and *SLC45A2* variant p.L374F (het)	The UCM might code for the hypomorphic allele, or the underlying genetic predisposition might be due to another OCA locus and not OCA1; again, the hypomorphic variant p. S192Y might contribute to the OCA1B phenotype
Goto 2004[25]	p.G97V; OCA1B	0/0	Total	IVS2−10deltt-7T>A	Yes	–	IVS2−10deltt-7T>A was found to disrupt normal splicing by functional analysis. The authors suggested that the relatively low expression of aberrant mRNA as identified in the functional analysis could imply that this mutation generated the mild phenotype OCA1B
Wang 2009[26]	p.K142M; OCA1B	0/0	Total	p.R299H[d]	Not reported	p.R299H has been reported as an OCA1A variant and our functional analysis showed null activity	Functional characterization suggests OCA1A phenotype, not consistent with OCA1B as reported in the Albinism Database. The variations could lie in the same allele and the variation in the other allele could be unidentified that might code for the hypomorphic variant
Hutton 2008[24]	p.H202R; OCA1A/B[b]	0/0	Total	p.D448N[d]	Not reported	p.D448N has been reported as an OCA1B variant but our functional analysis showed null activity	Reported clinical characterization as OCA1A explained, but their molecular characterization as OCA1B not supported. No functional work done in the study

(continued)

Table 3 (continued)

Study	Selected variant; OCA type	TH/DO activities (% of WT)	Level of ER retention	Second variation	Was segregation analysis done?	Comments	Possible explanation
King 2003[23]	p.P209R; OCA1A	0/0	Total	UCM	Yes	–	The UCM might code for the hypomorphic allele or the underlying genetic predisposition might be due to another OCA locus and not OCA1
Spritz 1997,[27] Hutton 2008[24]	p.T325A; OCA1B	90·7/82·3	Similar to WT	UCM	Not reported	Presence of the temperature-sensitive variant p.R402Q (rs1126809) (hom)	The underlying genetic predisposition might be due to another OCA locus and not OCA1
Badens,[a] King 2003[23]	p.Y327C; OCA1A	0/0	Total	UCM	Yes (King)	–	The UCM might code for the hypomorphic allele if OCA1, or the underlying genetic predisposition might be due to another OCA locus and not OCA1.
King 2003,[23] Opitz 2004[19]	p.M332T; OCA1B	0/0	Total	UCM in King study; p.V24F and p.G47V in Opitz	Yes (King)	In the Opitz study, where p.M332T was identified with two other variations: p.G47V and p.V24F, the OCA1 status was not given	The UCM in the King study might code for the hypomorphic allele if OCA1, or the underlying genetic predisposition might be due to another OCA locus and not OCA1
Passmore 1999[28]	p.N371Y; OCA1B[c]	0/0	Total	p.731–732delGT	Not reported	c.731–732delGT has been reported in a case of OCA1A	Functional characterization suggests OCA1A phenotype, not consistent with OCA1B as reported in the Albinism Database. The variants could lie in the same allele, and the variants in the other unidentified allele might code for the hypomorphic variant
King 2003[23]	p.R422W; OCA1B	0/0	Total	p.T373K[d]	Yes	p.T373K has been reported as an OCA1A variant and our functional analysis showed null activity	Functional characterization suggests OCA1A phenotype, not consistent with OCA1B as reported in the Albinism Database
Zahed 2005[29]	p.Y433C; OCA1B	44·5/32·2	Partial	Not reported	Not reported	–	p.Y433C could actually be an OCA1B variant

TH, tyrosine hydroxylase; DO, dopa oxidase; WT, wild-type; ER, endoplasmic reticulum; IVS, intervening sequence; UCM, uncharacterized mutation; hom, homozygous; het, heterozygous. All of the selected variants mentioned in the table were reported to be in the heterozygous state. [a]Unpublished mutation: Badens C, Centre d'Enseignement et de Recherche en Genetique Medicale Faculte de Medecine, 13385 Marseille CEDEX 5, France, 2001. [b]In the Hutton and Spritz study[24] the variant was designated as OCA1A and OCA1B through clinical and molecular diagnosis, respectively. [c]In the Passmore *et al.* study,[28] the patient harbouring N371Y was reported to have yellowish white hair colour, deep blue iris colour and white skin, and was and clinically designated as OCA2, but reported as an OCA1B variant in the Albinism Database. [d]These three variants were assessed for their enzyme activities, once we determined that the corresponding variant in the other allele does not code for the hypomorphic variant needed for the OCA1B phenotype.

variations revealed concordance with SIFT and PolyPhen-2 in most but not all cases. For instance, in the case of the p.P45T variant, while molecular data show null activity, SIFT predicts it to be 'tolerated'. Similarly, for the p.A266T variant, which has > 50% activity, PolyPhen-2 predicts it to be 'probably damaging'. These observations highlight that although these prediction tools are very useful in the case of proteins where biological activity cannot be easily measured, they have limitations; direct determination of biological activity is definitely a better option when feasible.

In a recent study, K and Purohit attempted to provide insight into the underlying molecular mechanism involved in OCA1A through homology modelling and *in silico* analyses.[22] In that study, the two variants (p.N371Y and p.T373K) that they had found to be most deleterious, as predicted by an *in silico* approach, showed null activity through functional analysis in our studies (Table 3). These variants were predicted to differ from the WT TYR in terms of solvation free energy, number of hydrogen bonds and number of salt bridges, which could lead to structural abnormalities.[22]

To summarize, our analysis with 34 missense variants of TYR revealed ER retention as the major cause of loss of enzyme activity, irrespective of the location of the variation in the protein. The amount of protein released from the ER barrier could be correlated with the enzyme activity. Our analyses revealed that for true genotype–phenotype correlation in the case of OCA1, variant screening must be complemented with functional validation. Our findings also suggests that missense SNPs in *TYR* in the dbSNP panel could actually represent variations directly causal to OCA1, or hypomorphic alleles that could contribute to variation in the pigmentation profile of a population.

Thus, using OCA1 as a model disease, our study demonstrates a compelling need for functional evaluation of suspect variant proteins for better correlation of the resulting disease phenotype with the underlying genotype. Furthermore, the study opens up an interesting prospect for the need to understand the molecular basis of ER retention of the variants, irrespective of the site of variation.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Assessment of the correlation between the endoglycosidase H-released fraction of tyrosinase and enzyme activity.

