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Genome-wide linkage analysis of an autosomal recessive hypotrichosis identifies a novel P2RY5 mutation

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Abstract

While there have been significant advances in understanding the genetic etiology of human hair loss over the previous decade, there remain a number of hereditary disorders for which a causative gene has yet to be identified. We studied a large, consanguineous Brazilian family that presented with sparse woolly hair at birth that progressed to severe hypotrichosis by the age of 5, in which 6 of the 14 offspring were affected. After exclusion of known candidate genes, a genome-wide scan was performed to identify the disease locus. Autozygosity mapping revealed a highly significant region of extended homozygosity (LOD score of 10.41) that contained a haplotype with a linkage LOD score of 3.28. Results of these two methods defined a 9 Mb region on chromosome 13q14.11-q14.2. The interval contains the *P2RY5* gene, in which we recently identified pathogenic mutations in several families of Pakistani origin affected with autosomal recessive woolly and sparse hair. After the exclusion of several other candidate genes, we sequenced the *P2RY5* gene and identified a homozygous mutation (C278Y) in all affected individuals in this family. Our findings show that mutations in *P2RY5* display variable expressivity, underlying both hypotrichosis and woolly hair, and underscore the essential role of P2RY5 in the tissue integrity and the maintenance of the hair follicle.

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Keywords

P2RY5; G-protein coupled receptor; autosomal recessive hypotrichosis; autosomal recessive woolly hair; variable expressivity

Introduction

The hair follicle (HF) is a complex, multilayered structure formed by reciprocal interactions between the epithelium and underlying mesenchyme. The HF is formed in several stages which begin with the induction of an epithelial placode by the mesoderm, the thickening and downward movement of the placode, and the interaction between the dermal papilla (DP) and overlying epidermal cells which facilitates the differentiation of those cells into the different structures of the mature hair follicle. The HF undergoes continual shedding and renewal during the hair cycle. After a hair is shed, interaction between the DP and the epidermal derived stem cells leads to the formation of a new HF in a process called anagen, after which it enters a resting state or catagen, and is later shed during telogen [1]. The complex processes of hair follicle morphogenesis are controlled by numerous genes and signaling interactions, many of which remain unknown.

Several hereditary human hair loss disorders have facilitated gene discovery efforts and led to the identification of genes integral to HF formation or maintenance. To date, the causative genes have been identified for several hereditary hair diseases, such as Atrichia with Papular Lesions (HR; OMIM 209500;[2–5], Congenital Alopecia with T cell immunodeficiency (WHN; OMIM 601705; [6]), Localized Autosomal Recessive Hypotrichosis/ Monilethrix (DSG4; OMIM 607903 and ;[7–11]), Congenital Hypotrichosis with Juvenile Macular Dystrophy (CDH3; OMIM 601553; [12; 13]), Ectodermal dysplasia, Ectrodactyly, and Macular dystrophy Syndrome (CDH3; OMIM 225280;[12; 14]), Hypotrichosis Simplex of the Scalp (CDSN; OMIM 146520; [15]), and an autosomal recessive form of hypotrichosis (LIPH; OMIM 604379;[16]).

Recently, we identified mutations in the *P2RY5* gene in several Pakistani families with autosomal recessive woolly hair (OMIM 278150). The *P2RY5* gene is a nested gene residing within intron 17 of the *RB1* gene and encodes a heptaspan transmembrane protein that belongs to an orphan G protein-coupled receptor (GPCR). We determined that the *P2RY5* protein is predominantly expressed in Henle's and Huxley's layers of the inner root sheath of the HF [17]. This gene has also been implicated in autosomal recessive hypotrichosis simplex (OMIM146520)[18; 19].

Here, we identified a consanguineous Brazilian kindred affected with a severe form of autosomal recessive hypotrichosis, in which 6 of the 14 offspring presented with this rare phenotype. We performed genome-wide SNP genotyping to screen for a disease locus in this family. We identified a novel candidate region of linkage on chromosome 13q14.11-q14.2. After ruling out several plausible candidate genes in the region, we identified a pathogenic homozygous mutation in the *P2RY5* gene.

Materials and Methods

Samples

The family is of Brazilian origin and presented with a severe form of autosomal recessive hypotrichosis affecting 6 out of 14 offspring of a consanguineous marriage. Affected individuals were born with woolly and sparse hair that gradually fell out over the first 5 years of life. Additionally, the phenotype varied by gender, with females being more

severely affected than males. Females had a complete absence of scalp hair, eyebrows and eyelashes while males presented with sparse scalp hair (Figure 1B and 1C). Papular lesions were not present and there were no systemic abnormalities. A scalp skin biopsy from an affected individual showed a decreased number of HF, a miniaturized HF and a thin hair shaft (Figure 1D and 1E). Informed consent was obtained and peripheral blood was collected from participants. A total of 15 individuals were genotyped for this study.

Genotyping

The Affymetrix GeneChip Human Mapping 10K or 10K 2.0 array was used to genotype 14 samples, including both parents and all available offspring. Sample preparation followed the Affymetrix 10K protocol, with minor alterations. Briefly, 250 ng genomic DNA from each sample was digested for 2.5 hours with *Xba*I, mixed with *Xba*I adapters and ligated with T4 ligase for 2.5 hours. A single primer designed within the adapters was used to amplify fragments ranging from 250–1000 bp; 6 PCR reactions were run for each sample to ensure an adequate amount of product for subsequent steps. The PCR products were purified with a QIAquick PCR purification kit and pooled, fragmented with DNaseI, and end-labeled with biotin.–Hybridization was performed by the Columbia University Gene Chip Facility.

To confirm the SNP genotyping results, microsatellite markers were placed within the candidate region. Genomic DNA from the family members was amplified by PCR using primers for eight polymorphic markers on chromosome 13q: D13S263, D13S1272, D13S1312, D13S168, D13S153, D13S273, D13S262, and D13S1325. The amplification conditions for each PCR were 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 7 min. PCR products were run on 8% polyacrylamide gels and genotypes were assigned by visual inspection.

DNA Sequence Analysis of Candidate Genes

Two candidate genes were screened for mutations prior to genotyping, HR and DSG4. Following linkage analysis, nine genes on chromosome 13 were prioritized as candidates: *TSC22D*, *klotho*, *SLC7A1*, *AK057244*, *KIAA085*, *TPT1*, *Lcp1*, *ITM2B*, and *P2RY5*. For each of the 11 genes, all exons and exon-intron boundaries were sequenced. Primers were designed using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) or as described previously [17]. PCR products were purified with Rapid PCR Purification System (Marligen BioScience, Inc.), and bi-directionally sequenced using BigDye technology on an ABI Prism 310 sequencer. Sequences were compared to consensus sequences available from public human genome databases (<http://genome.ucsc.edu>; <http://www.ncbi.nlm.nih.gov>). Primer sequences and PCR conditions are available upon request.

For the *P2RY5* gene, in order to screen for the mutation C278Y, a part of exon 3 of the gene was PCR-amplified using a forward primer 5'-AGCAGTAAGGACAATGTACCCAATCAGTCTCT-3', and a reverse primer 5'-GACACTTTTCACAGTTGAAGGAACT-3'. Note that the C>G substitution was introduced into the forward primer to generate an *A/wM* restriction enzyme site only in the PCR product from the wild type allele (shown in bold and underlined). The amplification conditions were 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 7 min. The PCR products were digested by the *A/wM* at 37°C overnight and analyzed on a 1.5% agarose gel.

Genotype Analysis

For the initial analysis, GCOS software (Affymetrix) was used for quality control analysis and data formatting. The Transmission Disequilibrium Test allowing for Error (TDTAE)

[20] with the recessive mode of inheritance was used for analysis of the SNP genotyping data. Further analysis was subsequently performed with the Genespring GT software (Agilent), including inheritance checking, haplotype inference, and data analysis. Because the disease presented in a consanguineous family and was not present in at least the previous two generations, autozygosity mapping was used to identify regions in the genome that are homozygous for alleles that are identical-by-descent and shared among affected individuals. Scores are reported for regions as “regional LODs”. Details about the algorithm can be found at the Agilent website (www.chem.agilent.com/cag/bsp/products/gsgt/Downloads/pdf/autozygosity.pdf).

Additionally, parametric linkage analysis was used to further refine the region identified by autozygosity mapping. SNPs were analyzed individually and as clusters of inferred haplotypes by multipoint linkage analysis under the assumption of a recessive mode of inheritance and complete penetrance of a disease allele with a population frequency of 0.001. This program utilized SNP position information from dbSNP build 115 and National Center for Biotechnology Information (NCBI) build 35 of the human genome. Reported information is derived from dbSNP build 125 and NCBI build 36.2.

In Silico Analysis

Data for inter-species nucleotide sequence conservation was obtained from the UCSC Genome Browser (<http://genome.ucsc.edu>). Homology models of P2RY5 were produced by the G Protein-Coupled Receptor Data Base (GPCRDB; www.gpcr.org).

Results

We analyzed a consanguineous Brazilian family with severe hypotrichosis. Two known candidate genes, *DSG4* and *HR*, were sequenced and excluded for mutations prior to beginning the genome-wide scan. Next, both parents and all available offspring were genotyped using the Affymetrix 10K or 10K 2.0 mapping array. The average signal detection rate was 99.6% and the average call rate was 96.7%. There are 9943 SNPs that are common to the two versions of the mapping array. Of these, 6628 SNPs were polymorphic in this Brazilian family. Initially, the transmission disequilibrium test (TDT) was used for analysis of the genotype data. A single candidate region was identified on chromosome 13q12.12-q14.13, spanning 23 Mb and containing approximately 160 known genes and transcripts.

Five genes were initially identified as potential candidates for the disease locus, based on expression patterns or sequence homology to known hair follicle proteins. For example, *TSC22D*, located at 13q14.11, is a TGF- β inducible clone [21] that is expressed in the developing chick feather bud [22], and murine HFs [23]. While *TSC22D* appeared to be a promising candidate due to its expression, sequencing of all exons and intron-exon boundaries did not reveal a mutation. Other candidates were screened and excluded, including *klotho* and *SLC7A1*. In mice, mutations in *klotho* lead to an early aging phenotype which includes reduced hair follicle density [24]. Many solute carriers (SLC gene family) have been associated with transport within the HF [25; 26]. The genes for two hypothetical proteins located in this interval, *AK057244* and *KIAA085*, were also sequenced. These proteins have similarity to trichohyalin, a structural component of the inner root sheath [27], yet neither gene contained mutations.

Further statistical analysis was performed in an attempt to more clearly define the genomic region linked to the phenotype. Autozygosity mapping revealed a single highly significant region of extended homozygosity on chromosome 13q14.11-q14.3 ($Z=10.41$). This region spans 12.9Mb and contains 50 genes as well as an additional 59 predicted transcripts.

Linkage analysis reduced the interval to 9.02 Mb on chromosome 13.14.11-q14.2 (Figure 2), and microsatellite data confirmed the SNP evidence (data not shown). Three additional genes in this region (*TPT1*, *Lcp1*, and *ITM2B*) were identified based on their expression patterns in the hair follicle [26] and were sequenced and found to have no mutations.

Following the exclusion of these eight candidate genes, we discovered mutations in the *P2RY5* gene in patients with autosomal recessive woolly hair (ARWH) that was sparse in some affected individuals [17]. Because we had demonstrated expression in the HF, and this gene is located within our interval of linkage, *P2RY5* was sequenced and a mutation was identified. Direct sequencing of *P2RY5* in affected individuals led to the identification of a homozygous G to A transition at position 833 in the coding sequence of the *P2RY5*, which results in cysteine to tyrosine substitution at codon 278, designated C278Y (Figure 3). A screening assay with the restriction enzyme *A/wM* showed that the C278Y allele co-segregated with the disease phenotype (Figure 4). We confirmed that this mutation is not reported in any of the public databases and additionally screened DNA from 100 unrelated unaffected control individuals (200 chromosomes) to rule out the possibility of a rare polymorphism.

Discussion

In this study, we analyzed a Brazilian family that presented with autosomal recessive hypotrichosis. We performed a genome-wide scan with a low density SNP genotyping chip (10K). Because of the consanguinity present in this kindred, three statistical tests were performed on the SNP genotype data. The TDT is a powerful method to detect a disease region disproportionately shared by affected offspring and is an appropriate choice because all affected children are expected to be homozygous for the disease allele. TDT analysis implicated a 23Mb region on chromosome 13q. Autozygosity mapping was first proposed more than two decades ago as an efficient method for mapping recessive traits [28]. The development of high-throughput mapping arrays has greatly facilitated implementation of this method. Autozygosity mapping was utilized in this study and identified a 12.9 Mb region that exhibited extended homozygosity ($Z=10.41$) and was contained within the interval defined by TDT analysis. Finally, parametric linkage analysis performed under a recessive mode of inheritance narrowed the interval to 9Mb on chromosome 13q14.11-q14.12 ($Z=3.28$). This region overlapped with one previously reported [19]. Candidate genes were initially prioritized on the basis of reported expression patterns and/or homology to HF proteins. A total of eight candidate genes were sequenced and excluded: *TSC22D*, *klotho*, *SLC7A1*, *AK057244*, *KIAA085*, *TPT1*, *Lcp1*, and *ITM2B*.

We recently reported several homozygous mutations in the *P2RY5* gene in Pakistani families affected with ARWH [17]. In that study, we had noted a range in hair phenotypes among the patients, which included sparse hair for some affected individuals. In addition, we showed that *P2RY5* is predominantly expressed in the inner root sheath of the HF, which plays a crucial role in supporting and anchoring the growing hair. This data prompted us to postulate that *P2RY5* mutations may also cause a severe sparse hair phenotype. Direct sequencing of the *P2RY5* gene revealed a missense mutation which results in a cysteine to tyrosine substitution at codon 278 (C278Y) (Figure 3). This nucleotide is highly conserved and the affected amino acid residue is within the seventh transmembrane domain (TM7) of *P2RY5*. Figure 5 illustrates the location of all coding mutations that have been reported in *P2RY5* relative to protein domains, and nucleotide sequence conservation as measured by percent homology among 17 species.

In order to determine the structural effect of the C278Y mutation, we examined two homology models of *P2RY5*, one based on the crystal structure of rhodopsin and the other

based on bacteriorhodopsin, produced by the G Protein-Coupled Receptor Data Base (GPCRDB; www.gpcr.org) (Figure 6). In both models, residue C278 is positioned mid-membrane in helix 7, with the side chain partially exposed to the lipid bilayer. The hydrophilic character of cysteine suggests that it will interact with protein groups rather than the acyl chains of lipids. Substitution with the much larger side chain of tyrosine could lead to folding defects, although other possibilities such as interference with conformational transitions necessary for signaling are also possible.

We had originally considered *P2RY5* as a candidate gene based on our expression studies and because we observed that some Pakistani ARWH patients also had sparse hair. The range of hair phenotypes displayed by these patients included not only differences in hair density, but also texture and pigmentation. While the hair texture of most patients was woolly, some affected individuals displayed a less severe, wavy phenotype. Although most patients had dark hair which is typical for this region, some patients had depigmented hair shafts. The tremendous variation in hair phenotypes was found within, as well as between, families.

Genetic research across a broad spectrum of disorders has identified disease mutations for which a single genotype leads to a diverse range of phenotypes for a single trait, even within individual families. Examples of this phenomenon have been well documented in both complex and monogenic diseases. Interestingly, *P2RY5* is not the first gene implicated in a hair disorder to display variable expressivity. In 2006, three independent research groups identified *DSG4* as the causative gene for autosomal recessive monilethrix [8; 9; 11]. At the time this finding was published, it was surprising because mutations in *DSG4* were initially identified in families that were segregating localized autosomal recessive hypotrichosis (LAH). It was postulated that in the previous LAH studies, weakly formed moniform hair may have been present and were undetected [29]. Similarly, following our identification of a mutation in *P2RY5* in this hypotrichosis family, we obtained a more thorough clinical history of the patients and discovered that they had indeed been born with woolly sparse hair that progressed to hypotrichosis by the age of 5. Both autosomal recessive monilethrix and ARWH display a wide range of phenotypic variability, and interestingly, for each of these diseases the phenotypic variability includes both hair shaft anomalies and hypotrichosis.

Recently, mutations in *P2RY5* were also identified in Saudi families with autosomal recessive hypotrichosis simplex [18]. In these families, the severity of the hair loss phenotype was variable between affected individuals even within a single family. In addition, although hair texture was not discussed in this report, the clinical pictures of some affected individuals show evidence for phenotypes that are consistent with woolly hair (Figure 1 in reference [18]). While there is variable expressivity in the Brazilian family that we report here, the hypotrichosis is more severe than reported in Saudi families [18].

We have recently demonstrated that mutations in the Lipase H (LIPH) gene are causative in some ARWH patients (unpublished data) in addition to hypotrichosis. *P2RY5* has been shown to be a LPA receptor [18], providing a link between these discoveries. Collectively, this data suggests a crucial role of the LIPH/LPA/*P2RY5* signaling pathway in HF development and hair growth.

In this study, we have shown that a novel mutation in the *P2RY5* gene underlies hypotrichosis and provide additional evidence that similar to *DSG4*, the *P2RY5* gene displays variable expressivity, causing a range of phenotypic variations in hair.

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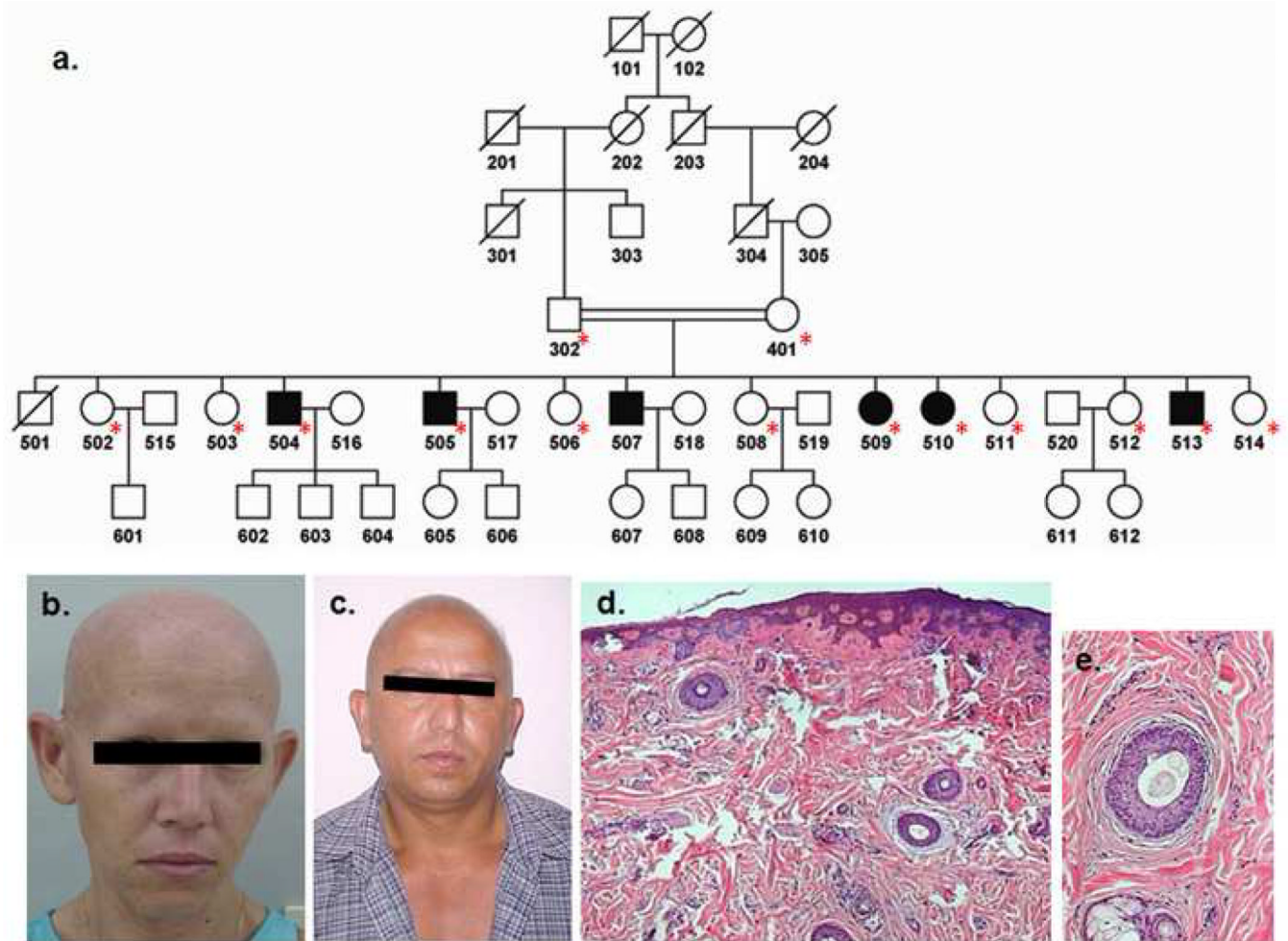


Figure 1.
 1A. Pedigree. The parents of the affected offspring are consanguineous. Red asterisks indicate samples that were genotyped with Affymetrix 10K arrays. 1B and 1C. Clinical presentation of the phenotype. 1B, females lack hair, eyebrows, and eyelashes; 1C, affected males display sparse scalp hair. 1D and 1E. Tissue biopsy viewed at 100X(1D) and 400X (1E). HF appear miniaturized and sparse. Despite an absence of hair, thin depigmented hairs appear in the follicles.

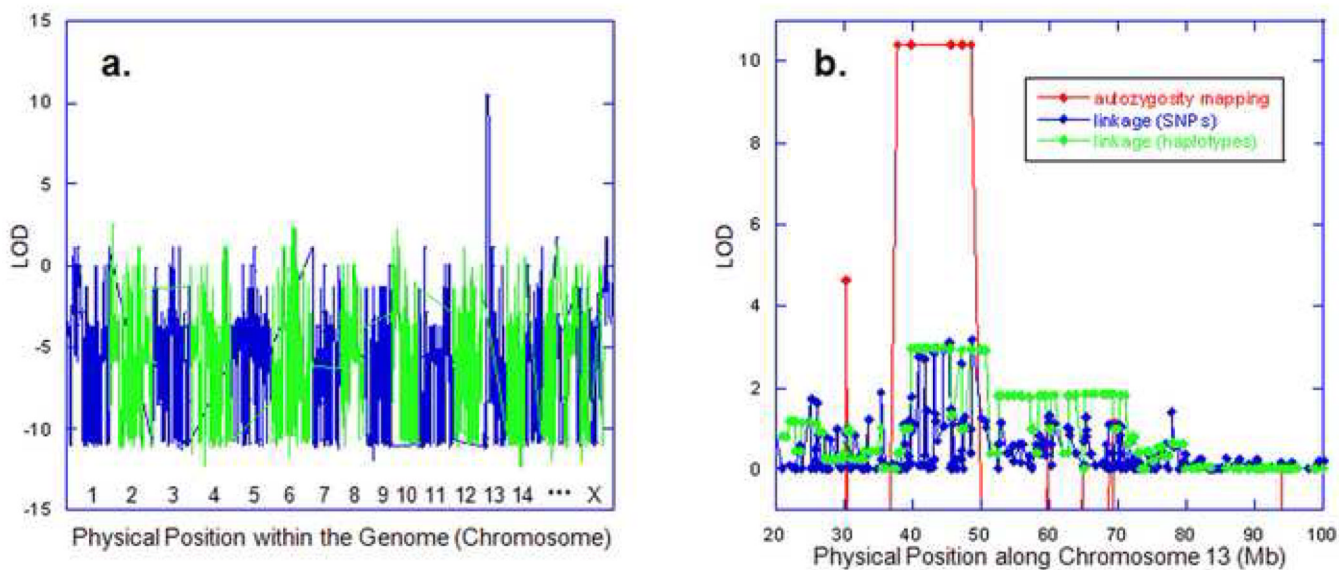


Figure 2. Results of Statistical Analysis. 2A. Autozygosity mapping results indicate a single highly significant region in the genome on chromosome 13, where alleles identical by descent are homozygous among affected individuals. 2B. Results of all statistical analysis on chromosome 13 SNP data. Parametric linkage analysis performed on individual SNPs (blue) and inferred haplotypes (green) under the assumption of a recessive mode of inheritance. Results of autozygosity mapping are plotted in red.

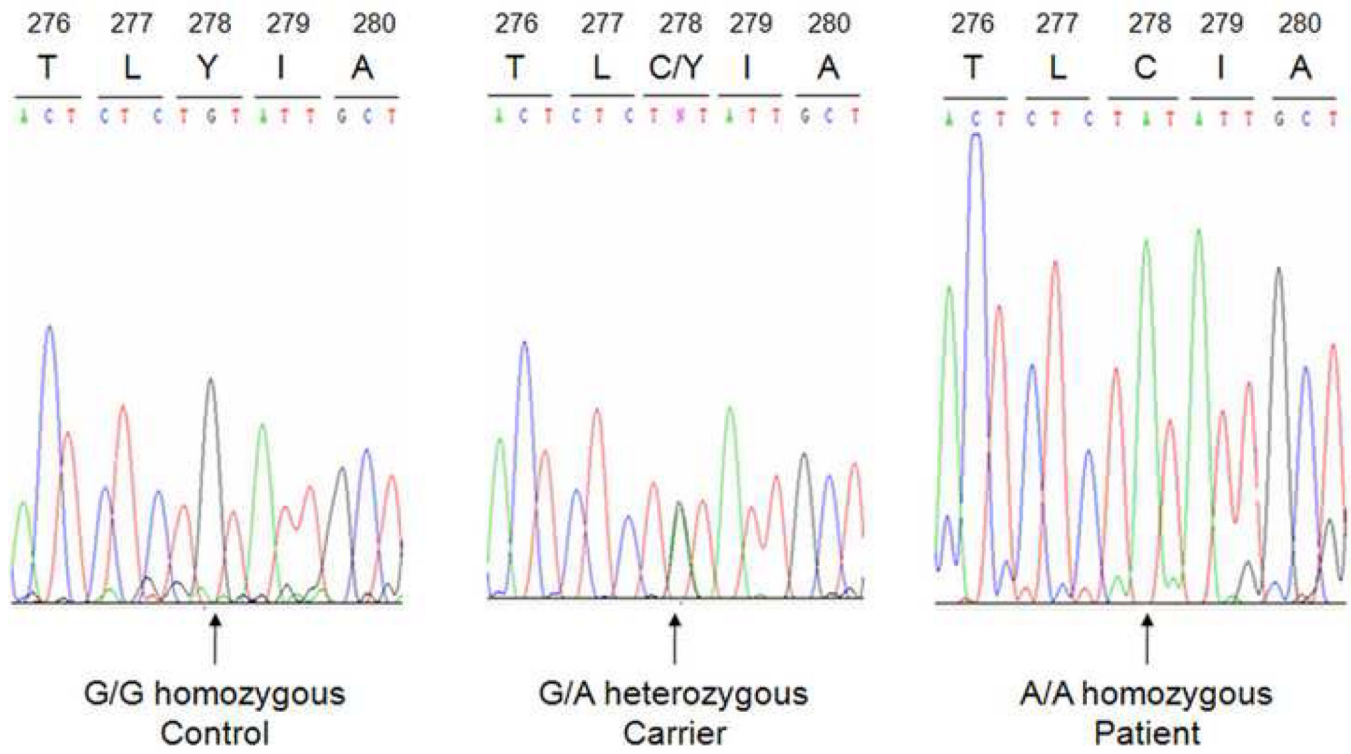


Figure 3. Identification of a homozygous mutation C278Y in the *P2RY5* gene.

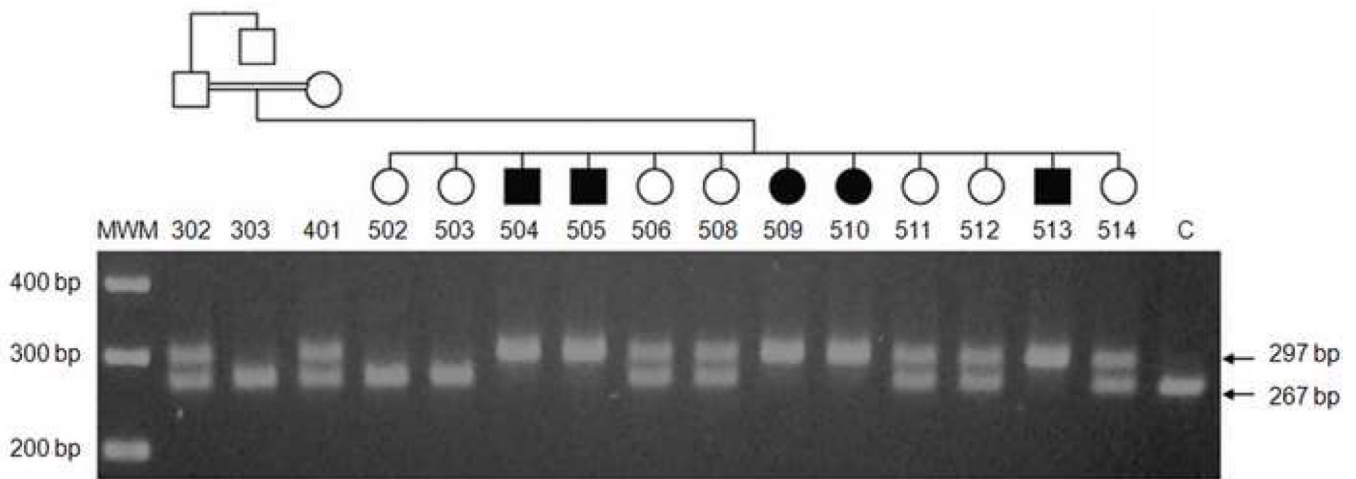


Figure 4.

Screening assay for the mutation C278Y. PCR product amplified using a mismatch primer, 297 bp in size, was digested with the *A/wM* restriction enzyme. The product from the wild type allele was digested into 267 bp and 30 bp fragments, while that from the mutant allele was undigested. The digested fragment, 30 bp in size, is not shown. The pedigree indicates relations and disease status of analyzed family members. C: control individual. MWM: molecular weight marker.

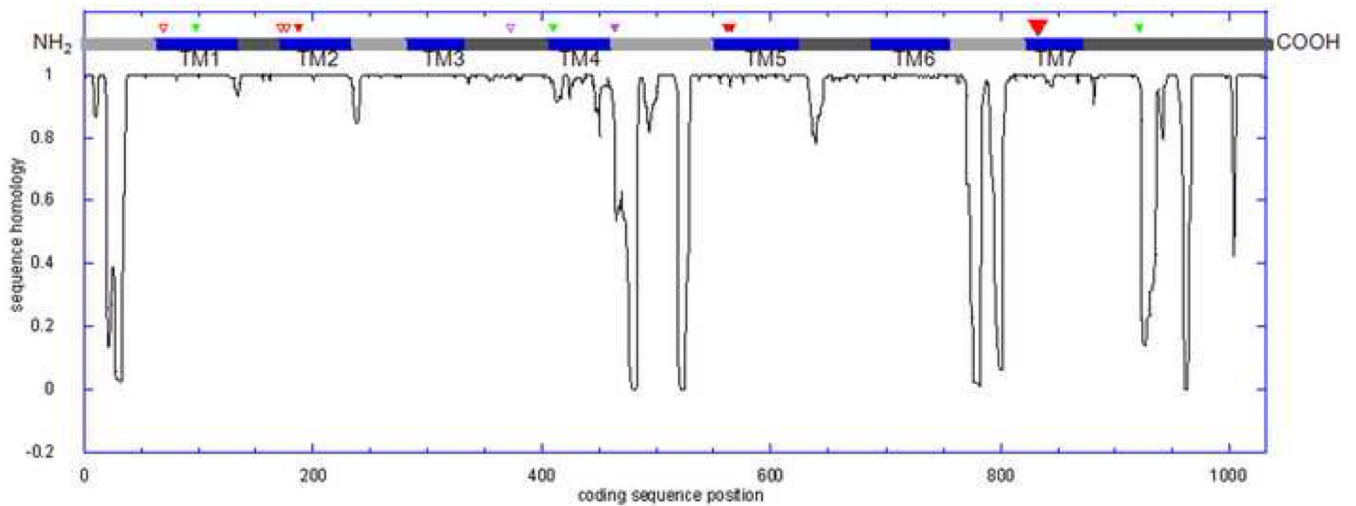


Figure 5.

The disease mutation identified in the Brazilian kindred (large red triangle) affects a nucleotide that is 100% conserved among 17 species, in the seventh transmembrane (TM7) domain. The location of all reported coding mutations in P2RY5 are displayed relative to the protein domains: extracellular domains (light grey), transmembrane domains (blue) and cytoplasmic domains (dark grey). Nucleotide sequence conservation is plotted below the protein model. SNPs reported in dbSNP are indicated by the green triangles. Mutations previously identified in families that segregate altered hair follicle phenotypes are indicated in red and purple triangles. Solid triangles indicate a nonsynonymous mutation, open triangles indicate a frameshift mutation.

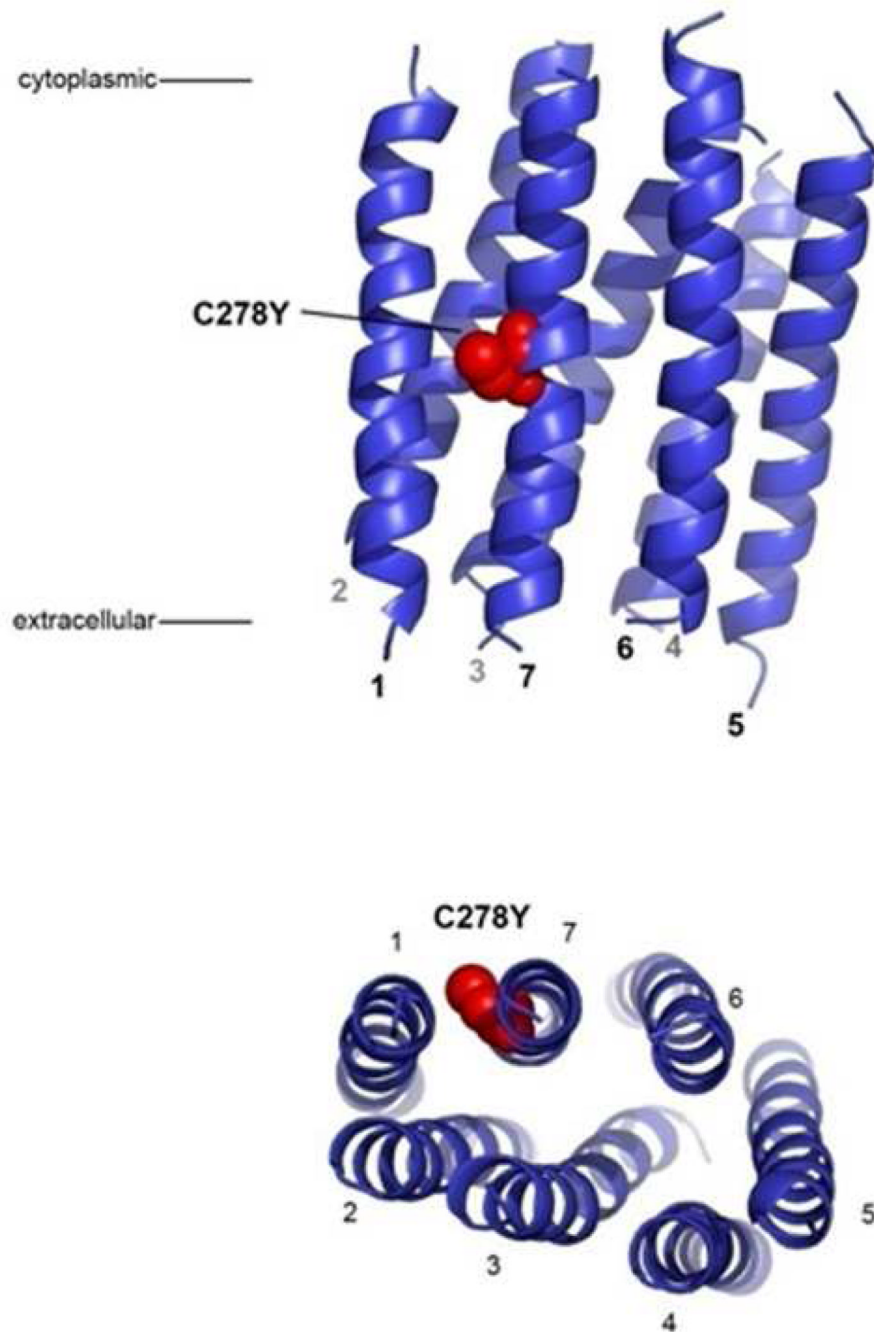


Figure 6. Structural positions of the P2RY5 missense mutation. A ribbon diagram is shown for a homology model of P2RY5 based on the crystal structure of rhodopsin. For the mutation, a space-filling representation of the native amino acid is shown in red. The transmembrane helices are numbered. Two orthogonal views are shown.