Genetic dissection of two Pakistani families with consanguineous localized autosomal recessive hypotrichosis (LAH)

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**Abstract**

**Objective(s):** Genetic analysis of two consanguineous Pakistani families with localized autosomal recessive hypotrichosis was performed with the goal to establish genotype-phenotype correlation.

**Materials and Methods:** Genomic DNA extraction had been done from peripheral blood samples. Extracted DNA was then subjected to PCR (polymerase chain reaction) for amplification. Linkage analysis was performed using the SCAP polymorphism linked to known alopecia loci. Family A excluded all known diseased regions that is suggestive of some novel chromosomal disorder. However, sequencing of P2RY5 gene in family B showed no pathogenic mutation.

**Results:** Both families were initially tested for linkage to known genes, which were involved in hair growth (5). Hair follicle is a complex mini-organ of human skin and it originates in complex morphogenetic interactions (6). Molecular investigation of consanguineous families proved to be a potent investigative tool to unveil these interactions by discerning multiple pathways in hair morphogenesis. Ahmad et al (1998) (7) reported the first case of alopecia with genetic disruption of the hairless (HR) gene on chromosome 8p21.1. Defect in HR gene resulted in atrichia with papular lesion in the affected individuals (8-10). Monilethrix hair loss and hypotrichosis (patch hair loss) with vesicular appearance on the skin were noted with disruption of desmoglein-4 (DSG4) and desmocollin (DSC3).

**Conclusion:** During past decade, application of homozygous mapping in consanguineous families with diverse hair loss phenotypes has improved the apprehension of disease pathogenesis at molecular level by exploring different physiological interactions involved in hair growth (5). Hair follicle is a complex mini-organ of human skin and it originates in complex morphogenetic interactions (6). Molecular investigation of consanguineous families proved to be a potent investigative tool to unveil these interactions by discerning multiple pathways in hair morphogenesis. Ahmad et al (1998) (7) reported the first case of alopecia with genetic disruption of the hairless (HR) gene on chromosome 8p21.1. Defect in HR gene resulted in atrichia with papular lesion in the affected individuals (8-10). Monilethrix hair loss and hypotrichosis (patch hair loss) with vesicular appearance on the skin were noted with disruption of desmoglein-4 (DSG4) and desmocollin (DSC3).

**Keywords:** Alopecia, Autosomal recessive, Hypotrichosis, Genetics, P2RY5 gene, Pakistan

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Institu showed typical males. Filled squares represent an affected individual.

Figure 3.1: Pedigree of Family A with hypotrichpsis. Circle s represent females and squares represent male and circles shows females. Filled squares show affected male individuals (Parallel horizontal lines represent consanguineous marriage)

Overlapping type of hypotrichosis phenotypes were noted with multiple genes/loci involvements that include lipase-H (LIPH) on chromosome 3q27 (15, 16). G-protein coupled receptor (LPAR6/P2RY5) on chromosome 13q14, and identification of loci with unknown genes on chromosome 1q11.23-22.237 (17) and chromosome 7p21.3-p22.3 (18) further elucidated both genotypic and phenotypic variability in the development of human hair.

In the present study, we have genotyped all known loci of two Pakistani families with hereditary hair loss. In order to get linkage support on known chromosomal regions we have used highly polymorphic markers.

Materials and Methods

Human subjects

The study was formally allowed by the Institutional Review Board (IRB) of National University of Science and Technology (NUST), Islamabad, Pakistan. The families (Figure 1 and Figure 2) showed typical features of hereditary hypotrichosis and were recruited from different areas within Pakistan. Their written consents were obtained and family pedigree was constructed from available information using a method introduced by Bennett et al. (19).

Genomic DNA isolation and genotyping

Genomic DNA was extracted from the venous blood samples using the standard phenol-chloroform method (20). PCR amplification of the extracted DNA was performed according to standard procedures using high polymorphic microsatellite markers for each locus (Table 1) (21). PCR products were loaded

| S. No. | Candidate genes / loci | Chromosomal location | Microsatellite markers * | Genetic location (cM) * |
|-------|------------------------|-----------------------|--------------------------|------------------------|
| 1     | Alopecia with mental retardation syndrome 1, 2 (APMR1, APMR2) & lipase H (LIPH) gene | 3q26.2-q27.3 | D3S1618 | 192.93 |
|       |                        |                       | D3S578  | 193.97 |
|       |                        |                       | D3S583  | 193.97 |
|       |                        |                       | D3S592  | 195.04 |
|       |                        |                       | D3S1617 | 196.4  |
|       |                        |                       | D3S1530 | 196.01 |
| 2     | Hairless (HR) gene     | 8p21.3                | D8S298  | 40.11  |
|       |                        |                       | D8S1786 | 41.41  |
|       |                        |                       | D8S1733 | 41.59  |
|       |                        |                       | D8S1752 | 42.55  |
| 3     | Gap junction proteins (GJB6 & GJB2) genes | 13q12.11 | D13S175 | 0.55   |
|       |                        |                       | D13S787 | 8.75   |
|       |                        |                       | D13S153 | 52.0   |
|       |                        |                       | D13S273 | 52.8   |
|       |                        |                       | D13S894 | 37.87  |
|       |                        |                       | D13S168 | 41.14  |
| 4     | Localized autosomal recessive hypotrichosis (LAH3) and P2RYS gene | 13q14.11-q21.32 | D13S1253D13S1227 | 44.56 |
|       |                        |                       | D13S153D13S1227 | 51.37 |
| 5     | Cadherin 3 (CHD3) gene | 16q22.1              | D16S107  | 86.57  |
|       |                        |                       | D16S1025 | 86.57  |
|       |                        |                       | D16S1095 | 86.57  |
|       |                        |                       | D18S478  | 50.5   |
|       |                        |                       | D18S847  | 53.28  |
|       |                        |                       | D18S1107 | 45.89  |
|       |                        |                       | D18S1177 | 51.98  |
|       |                        |                       | D18S36   | 53.29  |
|       |                        |                       | D18S457  | 55.14  |
|       |                        |                       | D18S456  | 55.74  |
|       |                        |                       | D18S894  | 60.1   |
| 6     | Desmogelins and desmocollins (DSG and DSC Cluster) | 18q21 | D18S298 | 18.57  |
|       |                        |                       | D18S299  | 18.57  |
|       |                        |                       | D18S300  | 18.57  |

Abbasi et al Genetic dissection of two LAH Pakistani families

Figure 1. Pedigree of family A, showing two affected individuals. The squares represent male and circles show females. Filled squares show affected male individuals.

Figure 2. Pedigree of family B, showing five affected individuals. The squares represent male and circles show females. Filled squares and circles represent affected individuals (Diagonai cross means dead individuals)
DNA sequencing

In order to search for pathogenic mutations, biallelic (bi-directional) sequencing was performed for the gene P2RY5 using Beckman Coulter 800. Exon and intron-exon boundaries of this gene were PCR amplified from genomic DNA using primers that were designed from intronic and exonic sequences. Primers were designed using Primer3 software (22) (Table 2). In order to get a complete chromatogram for a targeted DNA sequence, two thermocycling reactions (1st and 2nd PCR) and two purifications protocols (1st purification and 2nd purification) were performed. Each thermocycling reaction was followed by one purification protocol consecutively.

Results

Clinical findings

The study, presented herein, described clinical and molecular analysis of Pakistani families, with the same phenotype as hypotricosis simplex mostly linked with P2RY5 and LIPH genes (23, 24).

Table 2. Primers for PCR amplification of P2RY5 gene exon

| Primer                        | AMP Length (bp) | Annealing temp (°C) |
|-------------------------------|-----------------|---------------------|
| Forward primer                | Reverse primer  |                     |
| P2RY5                         |                 |                     |
| GCTCTCTGAAACCTCTGACAATG       | CAAGCTGACTACCAAAACG | 700 | 57 |
| GCAGCTGAGAAAAGTG              | CTAGCTAAAAGCCGTAAACG | 650 | 57 |
| GATGATTCTCTGCAATTGTC          | CTCATTCTTGTATCATGGCTG | 700 | 57 |

on 8% non-denaturing polyacrylamide gel by bromophenol blue as loading dye. The gel was further stained with ethidium bromide and genotypes were allotted by visual examination using UV gel doc system (BioRad).

The affected individuals of the family exhibited typical features of localized autosomal recessive hypotrichosis (LAH) including sparse scalp hair, absent eyebrows and eyelashes, absent auxiliary and body hair. At birth, hairs were present on the scalp but after ritual shaving, which is usually performed a week after birth, they re-grew sparsely after a few weeks. Affected individuals did not show any other abnormalities. Ages of the affected individuals ranged from 4 to 25 years at the time of study (Figure 3).

Genetic mapping of candidate genes for autosomal recessive alopecia

“Homozygosity mapping” method was adopted to identify the locus that concealed the candidate gene that was responsible for causing congenital hair loss in these families; and also to determine the diseased gene in families.

Both families were initially tested for linkage to known genes that were involved in human hereditary hypotrichosis by genotyping highly polymorphic microsatellite markers. This included microsatellite markers linked to alopecia with mental retardation syndrome-1 and -2 (APMR1, APMR2) and lipase H (LIPH) gene (D3S1564, D3S2328, D3S3520, D3S2427, D3S1754, D3S2314, D3S1571, D3S3609, D3S3592, D3S1617) on chromosome 3q26.2-q27.3; locus for autosomal recessive hypotrichosis on chromosome 7p21.3-p22.3 (D7S2474, D7S516, D7S2484, D7S462, D7S517, D7S511, D7S1492, D7S2478, D7S1527, D7S3047); Hairless (HR) gene (D8S298, D8S1786, D8S1733, D8S1752) on chromosome 8p21.3; locus for autosomal recessive hypotrichosis on chromosome 10q11.23-22.3 (D10S1772, D10S567, D10S1643, D10S549, D10S233, D10S502, D10S1647, D10S451, D10S1432, D10S1730); P2RY5/ LPAR6 gene (D13S1312, D13S168, D13S284, D13S1807) on chromosome 13q14.11-q21.32;

Figure 3. Clinical presentation of LAH3 phenotype. A) Phenotypic form of an affected individual (IV-5) of family B with thin hair on head and absent eyelashes and eyebrows; B) An affected individual (IV-6) of family B with sparse hair on scalp, eyelashes, and eyebrows

Figure 4. Electropherogram of an ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D13S1227 at 44.56 cM from P2RY5 candidate linkage interval at 13q14.12. The Roman numerals indicate generation number of the individual within the pedigree while their position in the generation is represented in Arabic numerals.
In the present study, two highly consanguineous families (A & B), demonstrating alopecia, were ascertained from different areas within Pakistan. In all these families alopecia was congenital and the mode of inheritance was autosomal recessive. The affected individuals of the families showed typical features of alopecia characterized by sparse/total absence of hair on scalp, sparse to absent eyebrows and eyelashes, and auxiliary and body hair (Figure 3).

Both families were tested for linkage by genotyping polymorphic microsatellite markers linked to known alopecia loci including autosomal recessive hypotrichosis (3q26.33-q27.2), G-protein coupled receptor gene, P2RY5 (13q12.11), corneodesmosin on chromosome (6p21.33), hairless gene (Bp21), desmosgin and desmocollin gene cluster (18q12.1).

The obtained results convincingly excluded the linkage to these loci in family A. This signifies that a novel gene is responsible for alopecia in this family. Therefore, it is suggested that in order to identify the responsible gene for primary alopecia in this family, genome wide search may be carried out with markers located on 22 autosomes.

On the other hand, genotyping analysis in family B showed linkage to P2RY5 locus on chromosome 13q14.11. Linkage to P2RY5 predicts that a gene mutation is responsible for primary alopecia.

Discussion

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alopecia in the affected individuals of this family. Analysis of genotyping results obtained from polymorphic microsatellite markers in P2RY5 candidate linkage interval revealed that markers D13S1227 and D13S153 located between 44.56cM to 50.00 cM were homozygous in the affected individual but heterozygous in the normal individual of the family members. This shows linkage in family B to P2RY5 locus on chromosome 13q14.12 where P2RY5 gene belongs to a family of purinergic nucleotide receptors that are coupled to G-proteins (27). The P2RY5 gene encodes 344 amino acids of P2Y5 protein (28). This contains four potential extracellular domains, four cytoplasmic domains and seven predicted hydrophobic trans-membrane regions (http://au.expasy.org/uniprot/P43657).

To date, a number of mutations have been detected in P2RY5 causing LAH3; e.g. c.436G>A (p.G146R) a missense mutation; c.361insA (p.D120fsX16) a frameshift mutation and stop at codon 16; c.460insA (p.S154fsX58) a frameshift mutation and stop at codon 58; c.462-5G>C (p.S3T) a missense mutation (22); c.565G>A (p.E189K) a missense mutation; c.691insCATG (p.24insHfs52) a frame shift mutation and stop at codon 52; c.188A>T (p.D63V) a missense mutation (2).

Sequence analysis of P2RY5 gene revealed no pathogenic sequence variants. The sequence variant from an affected individual of family B had also been compared with the previous known reported mutations (2, 4, 22). Thus, data suggest that there may be any pathogenic variant in either regulatory regions of P2RY5 or some other chromosomal region with strong linkage to autosomal recessive hypotrichosis in family B. Therefore, it is suggested that in order to identify the gene responsible for autosomal recessive hypotrichosis in this family, genome wide search will be carried out with markers located on 22 autosomes. As this family also showed linkage to D13S787 at 8cM on the same chromosome 13, therefore it is suggested that genotyping SNP markers or closely saturates microsatellite markers both upstream and downstream region of the linked interval will be performed in order to confirm novel loci involved in this family.

### Conclusion

The present research work propounds that there may be other genes involved in this locus with similar overlapping type of phenotypes in Pakistani population. It is strongly suggested that more affected families should be investigated to further enhance the scope of known genes for future research work in this area.

Therefore, after finding such mutations, modulating gene expression might be an interesting therapeutic approach to help address these hereditary disorders.

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