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Homozygosity Mapping in 8 Pakistani and Kashmiri Families for Known Alopecia Genes/Loci

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Abstract: The objective of the present study was to search for known alopecia genes/loci in highly inbred Pakistani and Kashmiri families. The term alopecia is broadly used for various forms of hereditary human hair loss, with large differences in age of inception, severity and coupled ectodermal abnormalities. Eight families with typical features of alopecia were included in the study and through linkage analysis, three of them were found linked with HR at 8p21.3, linkage interval at 12q21.2-q22 (containing several potential candidate genes) and ARWH1 locus (P2RY5) at 13q14.2 respectively. DNA sequence analysis in linked families revealed a C to G transition at nucleotide position 3978 (c. 3978C>G) in exon 18 of HR gene and a previously reported mutation, a homozygous 4 bp CATG insertion at nucleotide position 69 (c.69insCATG) in P2RY5 gene. The majority of the families which remained unlinked with markers for known alopecia genes highly signify the presence of novel gene/genes in causing the disease in these families and therefore, It is highly recommended that genome wide scan with microsatellite markers be performed to map the causative gene/genes.

Key words: Alopecia, linkage analysis, microsatellite markers, sequence analysis

INTRODUCTION

Hair loss in humans is a complex phenomenon, resulting from both genetic and environmental factors (Pasternack *et al*., 2008). It has been estimated that over 100 genes are expressed in the hair (Irvine and Christiano, 2000). Although hair disorders do not put life into danger, the way they negatively influence the social status and psychology of the affected individuals can not be neglected (Cotsarelis and Millar, 2001). Alopecia is the term collectively used for various forms of hereditary human hair loss (Ahmad *et al*., 1998), with large differences in age of inception, severity and coupled ectodermal abnormalities (Zlotogorski *et al*., 1998). Alopecia or hair loss is broadly categorized as non-syndromic, with no associated anomalies and syndromic, connected with defects in ectodermal and other structures (Wali *et al*., 2007a). Similarly, classification is also based on conditions with normal hair follicle but abnormal hair growth cycle and disorders with damaged hair follicle (Thiedke, 2003).

Isolated forms of hair loss includes hypotrichosis Simplex (MIM 605389) at 6p21.3, Marie Unna Hereditary Hypotrichosis (MIM 146550) at 8p21, congenital atrichia (MIM 203655) at 8p21, localized hereditary hypotrichosis (LAH, MIM 607903) at 18p12.1, autosomal recessive hereditary hypotrichosis (LAH2 and LAH3) at 3p26.33 and 13q14.11-q21.32, respectively (Rafique *et al*., 2003; Azeem *et al*., 2008; Naz *et al*., 2009). Five of these conditions hypotrichosis simplex, congenital atrichia, LAH, LAH2, LAH3 are known to be caused by mutations in corneodesmossin (CDSN, MIM 602593), hairless (HR, MIM 225060), desmoglein 4 (DSG4, MIM 607892) genes, Lipase H (LIPH, MIM 607365) and G Protein Coupled Receptor (GPCR) (P2RY5, MIM 609239) respectively (Azeem *et al*., 2008; Naz *et al*., 2009; Kljuic *et al*., 2003; Gilliam *et al*., 2003; Rafique *et al*., 2004; John *et al*., 2006a; John *et al*., 2006b). Mutations in CDH3 (MIM114021), a gene encoding a classical cadherin molecule, lead to a recessive disorder with associated abnormality known as hypotrichosis with juvenile macular dystrophy (HJMD; MIM 601553) (Perez-Moreno *et al*., 2003).

Due to the high rate of consanguineous marriages in Pakistani and Kashmiri population, it is necessary to find the consequences in terms of genetic anomalies including hereditary alopecia in order to make a suitable strategy for carrier screening and genetic counseling in these populations. For this purpose, we performed linkage analysis with makers for known autosomal recessive hair loss genes/loci on 8 families of Pakistani and Kashmiri origin and the linked families were further set to mutational analysis. The present study is aimed to provide valuable information about the prevalence of hereditary

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Table 1: List of microsatellite markers used for linkage analysis.

S. No.	Candidate Genes	Chromosomal Location	Markers	Distance (cM)*
1	Lipase H	3q26.33	D3S2314	190.04
	(*LIPH*)	3q27.1	D3S3578	193.97
		3q27.2	D3S1530	196.01
2	Hairless	8p22	D8S1731	30.41
	(*HR*)	8p21.3	D8S1116	41.9
		8p21.3	KW218	42.82
		8p21.3	D8S1734	45.12
3	Linkage interval containing	12q21.33	D12S351	101.87
	VEZT, *KITLG*, *KERA* and *DUSP6* gene	12q22	D12S1345	103.49
		12q22	D12S327	106.19
		12q23.1	D12S1063	111.79
4	G-protein coupled purinergic receptor (*P2RY5*)	13q14.13	D13S1312	48.03
		13q14.2	D13S168	51.37
		13q14.2	D13S287	51.7
		13q14.2	D13S118	52
		13q14.2	D13S273	52.8
		13q14.3	D13S1807	55.5
		13q21.1	D13S1301	55.64
5	Cadherin 3 (*CDH3)*	*16q12.2*	D16S3034	67.57
		16q21	D16S3050	84.8
		16q21	D16S3019	85.69
		16q22.1	D16S3085	86.57
		16q22.1	D16S3095	87.61
6	Desmoglein 4 (*DSG4)*	*18q12.1*	D18S47	54.83
		18q12.1	D18S536	56.29
		18q12.2	D18S1160	58.08
		18q12.2	D18S1100	58.36

*: Average-sex distance in centiMorgan (cM) according to Rutgers combined linkage-physical human genome map

alopecia in highly inbred families and the chances of finding novel genes/loci, which will help to further explore the hereditary alopecia at molecular level.

MATERIALS AND METHODS

The present study consists of eight families with typical features of alopecia and was completed at Quaid-i-Azam University, Islamabad from February to September, 2010. Families were located in different areas like district Kohat, Hafizabad, Kupwara (Jammu and Kashmir), Thatha, and Bagh (Azad Jammu and Kashmir). The families were visited at their places of residence to generate pedigrees following a standard protocol (Bennett *et al*., 1995) and collect other relevant information. All participants gave their written informed consent to take part in the study. Photographs of each individual assessed to be affected were taken. Blood samples from affected and normal individuals of each family were collected for DNA extraction.

Linkage analysis: DNA extraction: Genomic DNA was extracted from bloods of study subjects following standard phenol-chloroform procedure (Sambrook *et al*., 1989) and after extraction DNA was dissolved in appropriate amount (150-200 μL) of Tris-EDTA (TE) buffer.

PCR amplification: Microsatellite markers of known alopecia loci (Table 1) were amplified by Gene Amp PCR system 9600 (Perkin Elmer, USA) and T3 thermocyclers (Biometra, Germany).

Genotyping: After amplification, the PCR products were loaded on 2% agarose gel (horizontal gel electrophoresis) and analyzed through UV transilluminator (Biometa, Germany) for confirmation. In the next step, genotyping was performed by first resolving the amplified PCR products in 8% standard non-denaturing polyacrylamide gel, performing the electrophoresis in a vertical gel tank (vertical gel electrophoresis) of Whatman Biometra (Biometra, Germany) and then visualization of markers through UV transilluminator (Biometra, Germany). Photographs were then taken with the help of a Digital camera DC 290 (Kodak, USA) for the genotypes to be assigned. Markers were numbered according to Microsatellite markers mapped by Cooperative Human Linkage Centre (CHLC) and were obtained from Invitrogen Genelink (USA). Information about the cytogenetic locations, size (in base pairs) and cM distance of the markers was obtained from UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway?org=Human&db=hg19&hgsid=175211401) and Rutger's map (Kong *et al*., 2004).

DNA sequencing: Three out of eight families initially showed linkage to markers for known alopecia loci/genes. To search for mutations of HR, VEZT and P2RY5 genes in family E, F and H, their respective exons were PCR amplified by Gene Amp PCR system 9600 (Perkin Elmer,

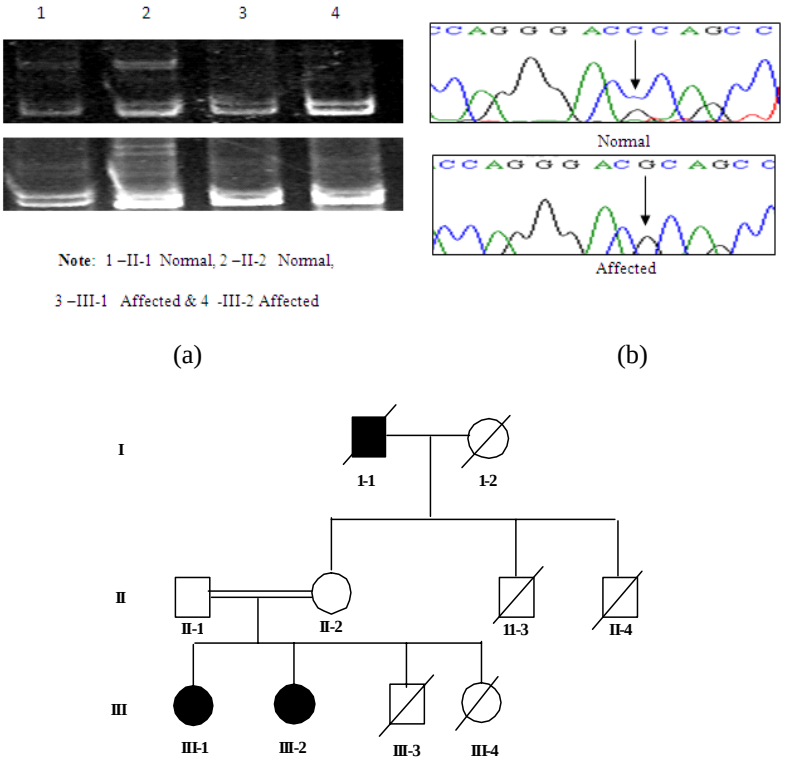


Fig. 1: (a) Electropherograms showing allele pattern obtained with markers KW218 (upper) and D8S1734 (lower) for *HR* locus in family E, (b) Sequencing chromatograms of exon 18 of *HR* gene, indicating a C to G transition at nucleotide position 3978 (c. 3978C>G). Arrows indicate position of the nucleotide change in the sequence, (c) Pedigree of family E with Alopecia

USA) from genomic DNA using specific primers. Commercially available kits (Fermentas Life Sciences) were used to purify the amplified products and sequenced directly on a CEQ8800 using the DTCS Quick Start sequencing kit (Beckman Coulter, USA) together with CEQ8800 DNA sequencer (Beckman Coulter, USA).

Mutation screening: Mutation screening was performed by comparing the gene sequences of normal and affected individuals with the corresponding control gene sequences from Ensemble Genome Browser database (http://www.ensembl.org/index.html). Sequence variants were identified via BIOEDIT sequence alignment editor version 6.0.7.

RESULTS

Linked families:

Family E: This family is three generation pedigree (Fig. 1c) residing in Bagh district of Azad Jammu and Kashmir. The family consists of four individuals including two affected females (III-1, III-2) in the third generation and prefers to marry within their own community. The pedigree is strongly suggestive of autosomal recessive type of disorder as parents were normal indicating that they were carriers for congenital alopecia. No dysmorphic features other than complete hair loss with sparse eye brows and eye lashes was observed in affected individuals (Fig. 5e) and they had no associated abnormality.

Linkage analysis with markers for known loci/genes indicated that markers KW218 and D8S1734 (Fig. 1a), were homozygous in the affected individuals but heterozygous in the parents, thus showed the linkage in family E to HR locus on chromosome 8p21.3. DNA sequence analysis of HR gene revealed a C to G transition at nucleotide position 3978 (c. 3978C>G) in exon 18, converting amino acid Proline into Arginine (p. P1326D) (Fig. 1b).

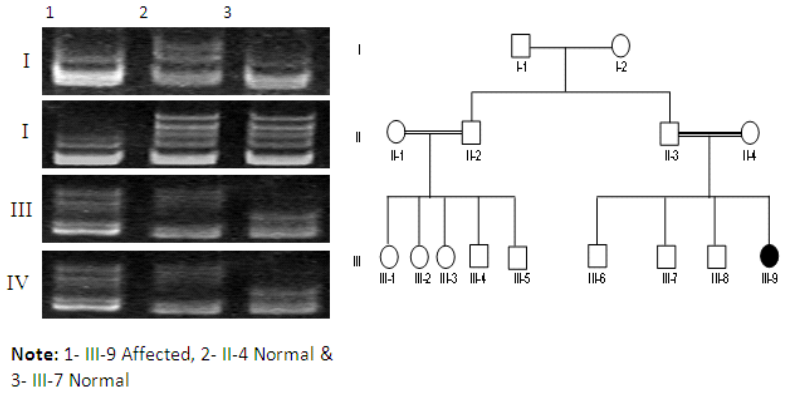
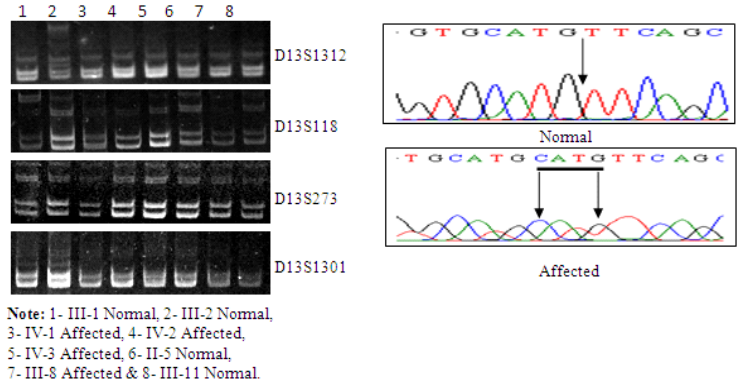
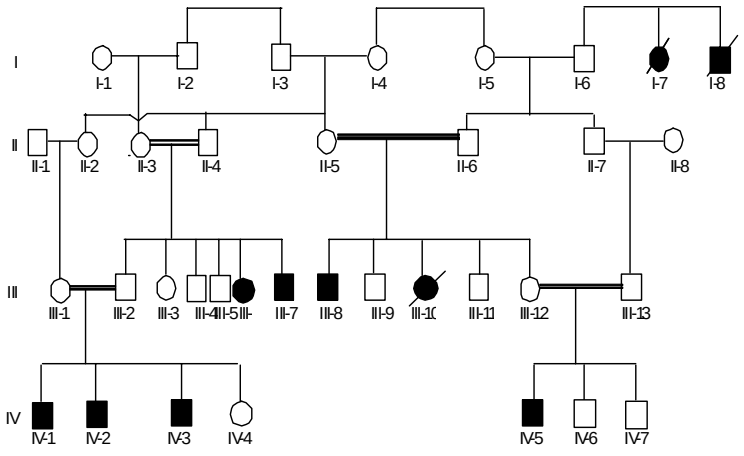


Fig. 2: (a) Electropherograms showing allele pattern obtained with markers D12S351 (I), D12S1345 (II), D12S327 (III) and D12S1063 (IV) in family F for linkage interval containing *VEZT*, *KITLG*, *KERA* and *DUSP6* gene, (b) Pedigree of family F with congenital alopecia



(a) (b)



(c)

Fig. 3: (a) Electropherograms showing allele pattern obtained with markers D13S1312, D13S118, D13S273 and D13S1301 in family H for *P2RY5* locus, (b) Sequencing chromatogram of single coding exon of *P2RY5* gene in normal and affected, indicating 4 bp insertion at nucleotide position 69, producing frame shift and resulted in premature stop codon 29 bp downstream (c.69insCATG). Arrows indicates the position of insertion in the sequence, (c) Pedigree of family H with congenital alopecia

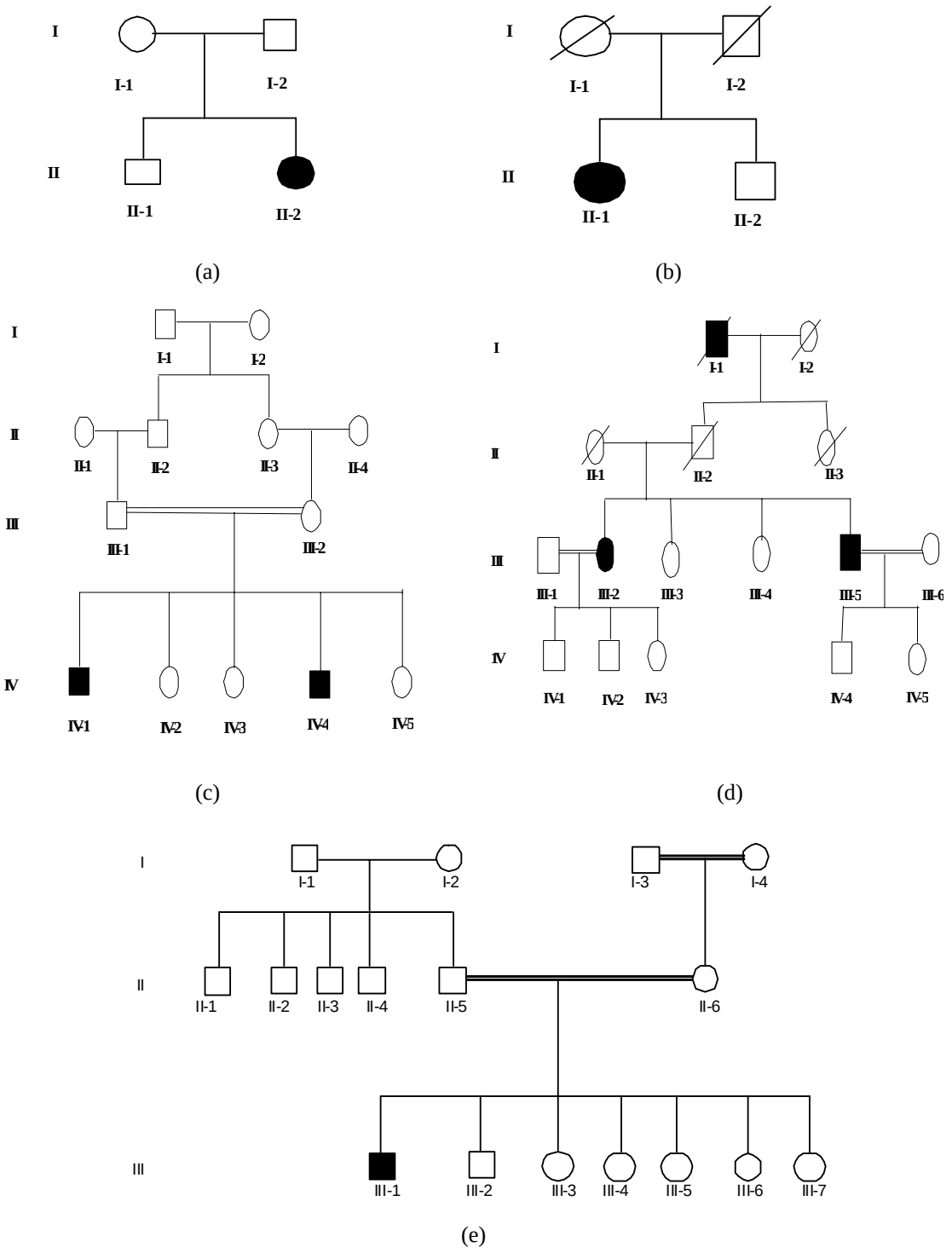


Fig. 4: Pedigrees of unlinked families: (a) Family A, (b) Family B, (c) Family C, (d) Family D and (e) Family G with Alopecia

Family F: Family F resides in Kohat district of khyber Pakhtunkhwa province and is a three generation pedigree (Fig. 2b) consisting of fifteen individuals, including only one affected female (III-9) in the third generation. When clinically examined, she was found to have complete alopecia of the scalp, sparse eyebrows and eyelashes

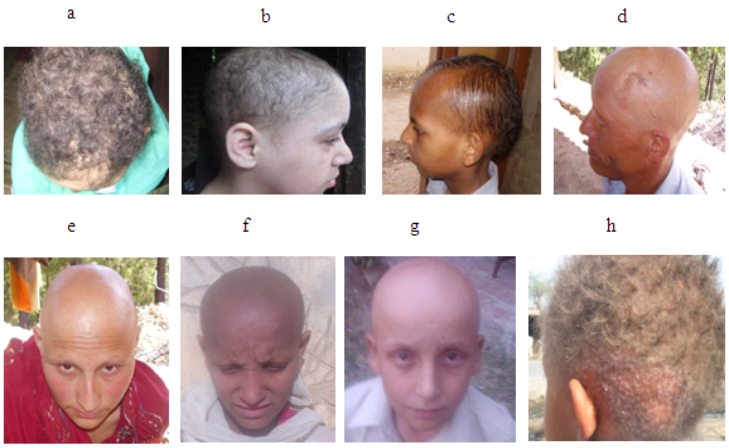


Fig. 5: Phenotypic appearance of the affected individuals of: (a) family A, (b) family B, (c) family C, (d) family D, (e) family E, (f) family F, (g) family G and (h) family H with alopecia

(Fig. 5f). The consanguineous parents and the other three brothers were normal indicating that the mode of inheritance is therefore autosomal recessive.

DNA analysis with polymorphic microsatellite markers D12S351, D12S1345, D12S327 and D12S1063 (Fig. 2a) showed these markers to be homozygous in the affected individual (III-9) but heterozygous in the normal ones, thus establishing linkage of family F to a region harboring *PLEKHG7*,*SLC6A15*, *VEZT*, *DUSP6*, *KERA* and *KITLG* gene on chromosome 12. A candidate gene vezatin (VEZT) was chosen. However, PCR-amplification of the entire coding region of VEZT showed no sequence variant in any of the 13 exons suggesting that some other candidate gene in this region must be responsible for the pathogenicity.

Family H: Family H resides in Hafizabad district of Punjab province. The family members because of strict social customs traditionally marry within their own community. The four-generation pedigree (Fig. 3c) shows thirty-six individuals including seven affected males (I-8, III-7, III-8, IV-1, IV-2 and IV-3) and three affected females (I-7, III-6 and III-10). Clinical history indicates typical hypotrichosis and wooly hair. There were no armpit hairs present although the pubic hairs were normal. Eyelashes and eyebrows were sparse in the affected individuals (Fig. 5h).

Eight DNA samples including four normal (II-5, III-1, III-2 and III-11) and four affected (III-8, IV-1, IV-2 and IV-3) individuals were used for genotyping and results showed that markers D13S1312, D13S118, D13S273 and D13S1301 (Fig. 3a) were homozygous in all the affected individuals but heterozygous in parents, thus establishing linkage of family H to P2RY5 at 13q14.2. Mutational analysis in affected individuals showed a homozygous 4 bp CATG insertion at nucleotide position 69 in P2RY5 gene, producing frameshift mutation and resulted in premature stop codon 29 bp downstream at codon 24 (Fig. 3b).

Unlinked families:

Family A: Family A was enrolled from Kupwara district of Jammu and Kashmir (India). Inter-marriages are a common practice here and the two generation pedigree (Fig. 4a) consists of four individuals, including one affected female (II-2). Analysis of pedigree is strongly suggestive of autosomal recessive mode of inheritance. Hypotrichosis was present by birth and affected individual was clinically normal with sparse woolly hairs and sparse auxiliary hairs (Fig. 5a). Age of the affected individual was 23 years at the time of study. Clinical examination was strongly suggestive of presence of a non-syndromic hypotrichosis in the family.

Family B: Family B resides in Kupwara district of Jammu and Kashmir (India) and prefers to marry within the family. This is a two-generation pedigree (Fig. 4b), composed of two individuals including one affected female individual (II-1). Clinical findings confirmed that the disease was congenital autosomal recessive hypotrichosis and was not associated to any environmental causes or part of a syndrome. The facial features of the affected individual were normal with sparse hair from birth (Fig. 5b).

Family C: Family C with congenital alopecia belongs to Thatha district of Sindh Province. The family members because of strict social customs traditionally marry within their own community. The four-generation pedigree (Fig. 4c) consists of 13members with two affected males (IV-1 and IV-4) in the fourth generation. Alopecia in the affected individuals was present by birth. No dysmorphic

features other than complete hair loss was observed. Affected individual was clinically normal, with sparse auxiliary hairs (Fig. 5c).

Family D: Family D resides in Bagh district of Azad Jammu and Kashmir The traditional system of marriages within the family/community resulted in high rate of consanguineous marriages. This is a four generation pedigree (Fig. 4d), which shows autosomal recessive mode of transmission of the disease. The affected individuals had normal facial features except complete hair loss with sparse auxiliary hairs and gap in teeth (Fig. 5d).

Family G: Family G was located in Kohat district of khyber Pakhtunkhwa Province and is a three generation pedigree (Fig. 4e), composed of sixteen individuals including one affected male individual (III-1). Consanguineous marriages within the family were common. Clinical features of the affected individual included complete alopecia of the scalp, sparse eyebrows and eyelashes (Fig. 5g).

In families A, B, C, D and G, analysis of the results obtained by genotyping polymorphic microsatellite markers (Fig. 5) linked to LIPH, HR, P2RY5, CDH3 and DSG4 candidate linkage intervals, excluded the families from linkage to any of the known loci. This indicates the involvement of novel genes in causing alopecia in these families.

DISCUSSION

Hair growth cycle mainly consists of a growth phase (anagen phase), regression phase (catagen) and resting phase (telogen) (Yu, 2006). Any disruption in the hair growth cycle, hair structure, signaling molecules and the pathways involved in developing hair follicles lead to hair abnormalities or alopecia (Ali *et al*., 2007). Among other hair disorders, atrichia with papular lesions (APL; OMIM 209500), due to defects in the hairless (HR) gene, is a rare form of irreversible alopecia inherited in autosomal recessive pattern with papular lesions of keratin-filled cysts on various regions of the body. APL is usually most common in consanguineous families residing in small geographical regions, but now it can also be seen at random in unrelated individuals (YIP *et al*., 2008). Hypotrichosis simplex (HS; MIM 146520, MIM 605389), a group of hereditary nonsyndromic isolated alopecias, causes hair loss which is usually visible at birth or in early childhood and progressing into adulthood (Sprecher, 2005). HS is inherited in both autosomal recessive and dominant manner. Mutations in CDSN lead to dominant HS, whereas autosomal recessive HS is caused by mutations in DSG4, P2RY5 and LIPH genes (Nahum *et al*., 2009). The P2RY5 is also known to cause autosomal recessive disease with wooly hair (ARWH1; MIM278150) (Shimomura *et al*., 2008a).

Consanguineous marriages are very common in Pakistani and Kashmiri population because of an integral part of their tradition attributing to the high incidence of alopecia cases in this population. In the present study, we linked three families E, F and H with HR at 8p21.3, linkage interval at 12q21.2-q22 (containing *PLEKHG7*, *SLC6A15*, *VEZT*, *DUSP6*, *KERA* and *KITLG* gene) and ARWH1 locus (P2RY5) on chromosome 13q14.2 respectively through homozygosity mapping with known alopecia genes/loci.

The hairless (HR; MIM602302) gene containing 19 exons codes for a putative zinc-finger transcription factor protein of 1189 amino acids that normally appears to be interacting with and influencing the transcriptional activity of several nuclear factors, including thyroid hormone receptor, retinoic acid receptor-related orphan receptor-a and vitamin D receptor (Potter *et al*., 2001; Hsieh *et al*., 2003). Mutations in HR are known to disrupt the regeneration of hair-follicle in mice and humans (Ahmad *et al*., 1998; Panteleyev *et al*., 1998). In the present study, family E ascertained from district Bagh of Azad Jammu and Kashmir showed linkage with markers KW218 and D8S1734 flanking HR locus on chromosome 8p21.3. The affected individuals displayed complete hair loss with sparse eye brows and eye lashes and they had no associated abnormality. DNA sequence analysis revealed a C to G transition at nucleotide position 3978 (c. 3978C>G) in exon 18, converting amino acid Proline into Arginine. Proline is normally present at ends of beta sheets and confers structural rigidity to protein conformation. Proline is non-polar amino acid that is converted into polar amino acid (Arginine) which may result in the disruption of HR gene function.

Affected individuals of family F (from district Kohat) showed complete alopecia with sparse eyebrows and eyelashes. Linkage in family F was established to chromosome 12q21.33-23.1 and haplotype analysis of this region also confirmed it. This region contains several potential candidate genes involved in hair growth including *PLEKHG7*, *SLC6A15*, *VEZT*,*DUSP6*,*KERA*and*KITLG*. More recently, Basit *et al*. (2010) reported a digenic inheritance at chromosomal regions 12q21.2-q22 (containing *PLEKHG7*, *SLC6A15*, *VEZT*,*DUSP6*,*KERA*and*KITLG gene*) and 16q21-q23.1 (containing CDH3 gene) in two consanguineous Pakistani families with hereditary autosomal recessive hypotrichosis. Mutational analysis of six candidate genes at 12q21.2-q22 didn't reveal any pathogenic variant, while DNA analysis of CDH3 gene at 16q21-q23.1 yielded a single base pair homozygous insertion (c.1024_1025insG and p.342insGfsX345) in exon 9 in one family and deletion of four base pair (c.1859_1862delCTCT and p.620delSfsX629) in exon 13

in another family. In our study, we choose VEZT gene for sequence analysis and we failed to detect any mutation in it. It is, however, estimated that family F in the present study might also be showing a digenic inheritance for hereditary hypotrichosis but needs further to be investigated.

Wali *et al*. (2007a) reported a third locus for autosomal recessive hypotrichosis (LAH3; MIM611452) through homozygosity mapping in consanguineous Pakistani families at chromosomal region 13q14.11-q21.32. Mutations in P2RY5 (MIM609239) has been ascertained to cause LAH3 (MIM611452) and autosomal recessive woolly hair (ARWH1MIM278150) (Petukhova *et al*., 2008; Wali *et al*., 2007a; Pasternack *et al*., 2008; Shimomura *et al*., 2008a). Since P2RY5 is expressed in suprabasal layers of epidermis and both Henle's and Huxley's layers of inner root sheaths (IRS) of hair follicle (Petukhova *et al*., 2008) probably support the notion that any defect in P2RY5 most probably leads to the disruption of IRS structure in hair follicles. In the present study, family H enrolled from district Hafizabad is showing linkage with ARWH1 locus at 13q14.2 with markers D13S1312, D13S118, D13S273 and D13S1301. Affected individuals exhibited typical features of hypotrichosis with woolly hair. Through Mutational screening in both normal and affected individuals, a previously reported mutation was found which is a homozygous 4 bp CATG insertion at nucleotide position 69 (c.69insCATG), producing frameshift mutation and resulting in premature stop codon 29 bp downstream at codon 24 (Shimomura *et al*., 2008b).

Majority of the families (A, B, C, D and G) studied in the project through genotyping with known markers didn't show any linkage to the established alopecia candidate regions. This signifies that novel gene/genes to be responsible for alopecia in these families. Therefore, it is suggested that in order to identify novel gene/genes responsible for alopecia in these families, genome wide search may be carried out with markers located on 22 autosomes.

CONCLUSION

The region of South Asia which includes Pakistan and Kashmir (Azad and Jammu), with a reasonably high rate of consanguineous marriages in diverse ethnic groups, is home to a wide variety of genetic disorders including alopecia. Although it provides a great source of information for the molecular dissection of an array of disorders, its negative effects in terms of social burdens are more than we can imagine. The majority of the families which remained unlinked with markers for known alopecia genes highly signify the presence of novel gene/genes in causing the disease in these families. It is highly recommended that genome wide scan with microsatellite markers be performed to map the causative gene/genes. An effort should be made to investigate more and more families with alopecia to find out the most common gene responsible for alopecia which will help to make a strategy for genetic counseling and carrier screening with most common genes for hereditary disorders in the region. It is very crucial that knowledge about consanguineous marriages and the associated risks should be made an integral part of social study subjects in the region, which will help to minimize the incidence of a wide range of genetic disorders prevailing in highly consanguineous families.

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