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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 8% polyacrylamide gel. Candidate gene was sequenced after gene linkage supported at highly polymorphic microsatellite markers of the diseased region.

Results: Both families were initially tested for linkage to known genes, which were involved in human hereditary hypotrichosis, by genotyping Highly polymorphic microsatellite markers. Family B showed partial linkage at *P2RY5* gene on chromosome 13q14.11-q21.32; hence, all exonic regions and their introns boundaries were subjected to DNA sequencing for any pathogenic mutation.

***Conclusion*:** Both families were tested for linkage by genotyping polymorphic microsatellite markers 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.

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Introduction

Hereditary alopecia shows discordant clinical features due to genetic impairment. It establishes itself in both autosomal dominant and autosomal recessive manners within human population. Worldwide reports have revealed that occurrence of such disorders are very low in populations where consanguinity is discouraged (1). However, in Pakistan, high rate of consanguinity have resulted in comparative increase in probability of the disease occurrence. Wholly hair loss is generally considered to be associated with *LPAR6* (P2Y5 protein) gene and hypotrichosis simplex is vividly found to be associated with *LIPH* gene (2-4).

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*),

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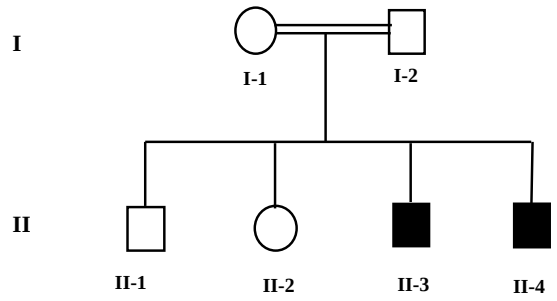


Figure 1. Pedigree of family A, showing two affected individuals. Squares represent male and circles shows females. Filled squares show affected male individuals
(Parallel horizontal lines represent consanguineous marriage)

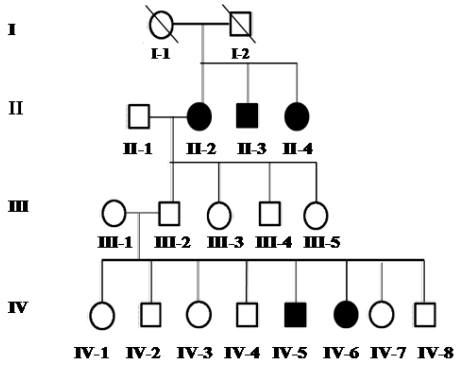


Figure 2. Pedigree of family B, showing five affected individuals. The squares represent male and circles shows females. Filled squares and circles represent affected individuals
(Diagonal cross means dead individuals)

respectively on chromosome 18q12 (11-14). 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 10q11.23-22.237 (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

Table 1. Microsatellite markers used to test linkage to candidate genes

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 D3S3578 D3S3583 D3S3592 D3S1617 D3S1530	192.93 193.97 193.97 195.04 196.4 196.01
2	Hairless (HR) gene	8p21.3	D8S298 D8S1786 D8S1733 D8S1752	40.11 41.41 41.59 42.55
3	Gap junction proteins (GJB6 & GJB2) genes	13q12.11	D13S175 D13S787 D13S153 D13S273	0.55 8.75 52.0 52.8
4	Localized autosomal recessive hypotrichosis (LAH3) and P2RY5 gene	13q14.11-q21.32	D13S894 D13S1253D13S1227 D13S168	37.87 41.14 44.56 51.37
5	Cadherin 3 (CHD3) gene	16q22.1	D16S3107 D16S3025 D16S3095	86.57 86.57 86.61
6	Desmogelins and desmocollins (DSG and DSC Cluster)	18q21	D18S478 D18S847 D18S1107 D18S877 D18S36 D18S457 D18S456 D18S384	50.5 53.28 45.89 51.98 53.29 55.14 55.74 60.1

Table 2. Primers for PCR amplification of P2RY5 gene exon

	5′→3′		AMP Length(BP)	Annealingtemp (°C)
	Forward primer	Reverse primer		
P2RY5	*GTCTCTGAAACTCTGCACTG*	*CAAGTCGTACCACAAACACG*	700	57
	GCAGCTGATGAAAGTGC	*CTAGCTAAAGACCGTTAACAG*	650	57
	GATCGATTTCTGGCAATTGTC	*CGTCATTCCTGTTACATGGGT*	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).

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).

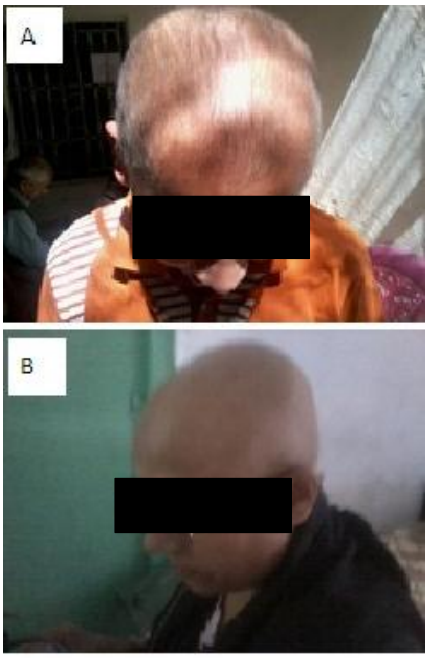


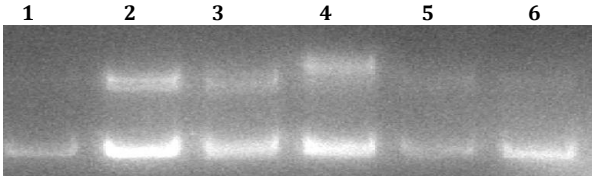
Figure 3. Clinical presentation of LAH3 phenotype. A) Phenotypic form of an affected individual (IV-5) of family B with thin hair on head and absent eyelashes and eyebrows; B) An affected individual (IV-6) of family B with sparse hair on scalp, eyelashes, and eyebrows

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

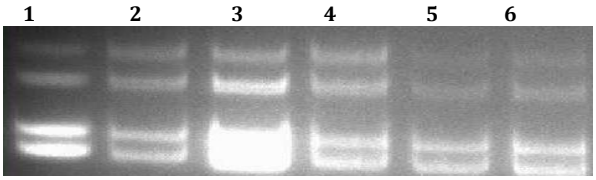
"Homozygositymapping" method was adopted to identify the locus that concealed the candidate gene that was responsible for causing congenital hair loss in these families; and also to determine the diseased gene in families.

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, D7S616, D7S2484, D7S462, D7S517, D7S511, D7S1492, D7S2478, D7S1527, D7S3047); Hairless (*HR*) gene (D8S298, D8S1786, D8S1733, D8S1752) on chromosome 8p21.3; locusfor autosomal recessive hypotrichosis on chromosome 10q11.23-22.3 (D10S1772, D10S567, D10S1643, D10S549, D10S2323, D10S502, D10S1647, D10S451, D10S1432, D10S1730); *P2RY5/ LPAR6* gene (D13S1312, D13S168, D13S284, D13S1807)on chromosome 13q14.11-q21.32;



1-IV-4 Normal 2-III-2 Normal 3-III-1 Normal
4-IV-2 Normal 5-IV-5 Affected 6-IV-6 Affected

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



1-IV-4 Normal 2-III-2 Normal 3-III-1 Normal
4-IV-2 Normal 5-IV-5 Affected 6-IV-6 Affected

Figure 5. Electropherogram of an ethidium bromide stained 8% non-denaturing polyacrylamide gel showing allele pattern obtained with marker D13S153 at 50.00 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

Cadherin 3 (*CHD3*) gene (D16S3107, D16S3025, D16S3095) on chromosome 16q22.1; and Desmogleins (*DSG*s) and Desmocollins (*DSC*s) gene cluster and Alopecia with Mental Retardation Syndrome-3 [APMR3 (D18S877, D18S847, D18S36, D18S456, D18S57)] on chromosome 18q11.2-q12.1.

This genetic testing proved the above-mentioned microsatellite markers good for Pakistani families, as every population is having its own set of associations and informative microsatellite (25, 26).

Family B showed partial linkage to *P2RY5* gene on chromosome 13q14.11-q21.32 (Figure 4 and Figure 5). As *P2RY5* gene has already been proven to be associated with autosomal recessive hypotrichosis, weak linkages can be ignored. Hence, all exonic regions and their introns boundaries were subjected to DNA sequencing for any pathogenic mutation (Figure 6).

Discussion

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 (8p21), desmoglin and desmocolin 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 chromosome13q14.11. Linkage to *P2RY5* predicts that a gene mutation is responsible for primary

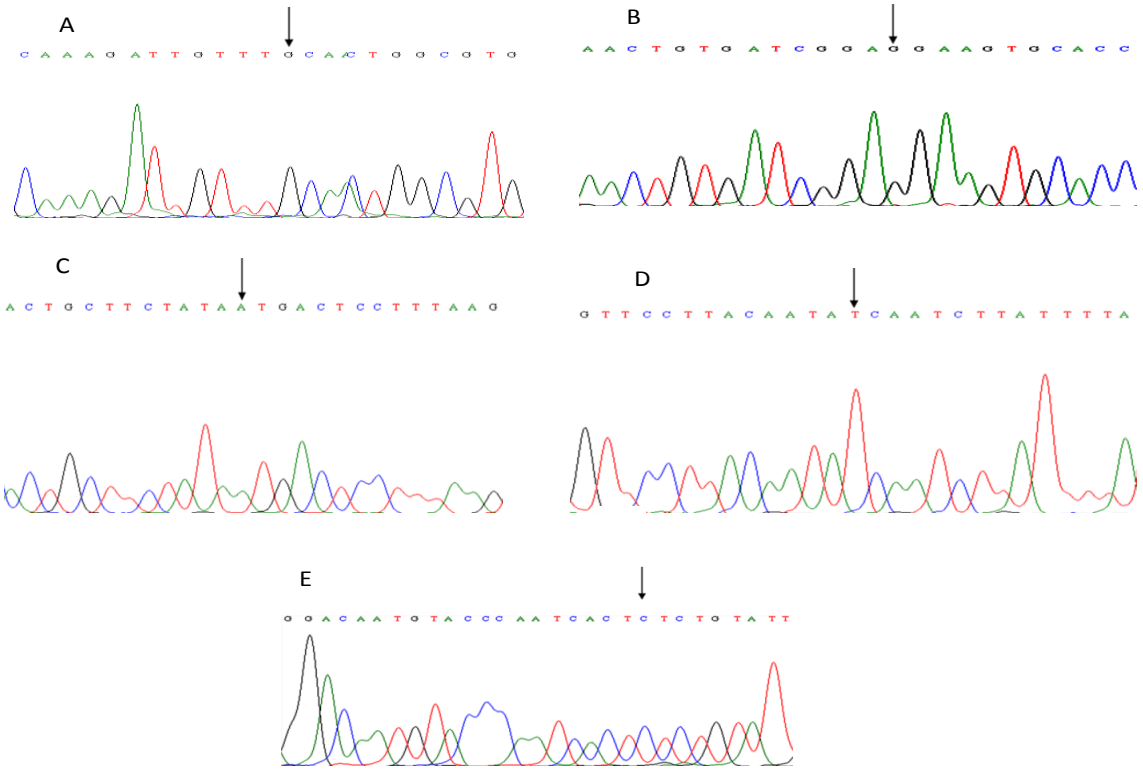


Figure 6. Sequencing chromatograms of *P2RY5* gene from an affected individual (IV-6) of family A indicating normal sequences at nucleotide positions 436(A), 35(B), 742(C), 830(D), and 463(E)

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 purinic 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.36insA (p.D13RfsX16) a frame-shift mutation and stop at codon 16; c.160insA (p.N54TfsX58) a frameshift mutation and stop at codon 58; c.8G>C (p.S3T) a missense mutation (22); c.565G>A (p.E189K) a missense mutation; c.69insCATG (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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References

1. Modell B, Darr A. Science and society: genetic counselling and customary consanguineous marriage. Nat Rev Genet 2002; 3:225-229.
2. Shimomura Y, Wajid M, Ishii Y, Shapiro L, Petukhova L, Gordon D, *et al*. Disruption of *P2RY5*, an orphan G protein-coupled receptor, underlies autosomal recessie woolly hair. Nat Genet 2008; 40:335–339.
3. Pasternack SM, Von Ku G, Aboud KA, Lee YA, Ruschendorf F, Voss K, Hillmer A , *et al* . G protein-coupled receptor P2Y5 and its ligand LPA are involved in maintenance of human hair growth. Nat Genet 2008; 40:329–334.
4. Tariq M, Ayub M, Jelani M, Basit S, Naz G, Wasif N, *et al*. Mutations in the *P2RY5* gene underlie autosomal recessive hypotrichosis in 13 Pakistani families. Br J Dermatol2009; 160:1006-1010.
5. Alkuraya FS . Homozygosity mapping: one more tool in the clinical geneticist's toolbox. Genet Med 2010; 12:236-239.
6. Millar SE. Molecular mechanisms regulating hair follicle development. J Invest Dermatol 2002; 118:216–225.
7. Ahmad W, FaiyazulHaque M, Brancolin V, Lam H, Aita V M, Owen J, de Blaquiere M, *t al* . Alopecia universalis associated with a mutation in the human hairless gene. Science 1998; 279:720–724.
8. Sprecher E, Lestringant GG, Szargel R, Bergman R, Labay V, Frossard PM, *et al*.. Atrichia with papular lesions resulting from a nonsense mutation within the human hairless gene. J Invest Dermatol 1999; 113:687-690.
9. Bale SJ. Of hairless mice and men: the genetic basis of congenital alopecia universalis/congenital atrichia. J Cutan Med Surg 1999; 3:309-311.
10. Cichon S, Kruse R, Hillmer AM, Kukuk G, Anker M, Altland K,*et al* .A distinct gene close to the hairless locus on chromosome 8p underlies hereditary Marie Unna type hypotrichosis in a German family. Br J Dermatol 2000; 143:811-814.
11. Kljuic A, Bazzi H, Sundberg JP, Martinez-Mir A, O'Shaughnessy R, Mahoney MG, *et al* . Desmoglein 4 in hair follicle differentiation and epidermal adhesion: evidence from inherited hypotrichosis and acquired pemphigus vulgaris. Cell 2003; 113:249-260.
12. Ayub M, Basit S, Jelani M, Ur Rehman FS, Iqbal M, Ahmad W. A homozygous nonsense mutation in the human desmocollin-3 (DSC3) gene underlies hereditary hypotrichosis and recurrent skin vesicles. Am J Hum Genet 2009; 85:515–520.
13. John P, Tariq M, ArshadRafiq M, Amin-Ud-Din M, Muhammad D, Waheed I, *et al.*Recurrent intragenic deletion mutation in desmoglein 4 gene underlies autosomal recessive hypotrichosis in two Pakistani

families of Balochi and Sindhi origins.Arch Dermatol Res 2006; 298:135-137.
14. Bazzi H, Getz A, Mahoney MG, Ishida-Yamamoto A, Langbein L, Wahl JK 3rd,*et al.*Desmoglein 4 is expressed in highly differentiated keratinocytes and trichocytes in human epidermis and hair follicle. Differentiation 2006; 74:129-40.
15. Kazantseva A, Goltsov A, Zinchenko R, Grigorenko AP, Abrukova AV, Moliaka YK, *et al.*Human hair growth deficiency is linked to a genetic defect in the phospholipase gene *LIPH*. Science 2006;314:982-985.
16. Aslam M, Chahrour MH, Razzaq A, Haque S, Yan K, Leal SM,*et al.* A novel locus for autosomal recessive form of hypotrichosis maps to chromosome 3q26.33–q27.3. J Med Genet 2004; 41:849–852.
17. Naz G, Ali G, Naqvi SK, Azeem Z, Ahmad W. Mapping of a novel autosomal recessive hypotrichosis locus on chromosome 10q11.23–22.3.Hum Genet 2010; 127395-401.
18. Basit S, Wali A, Aziz A, Muhammad N, Jelani M, Ahmad. Digenic inheritance of an autosomal recessive hypotrichosis in two consanguineous pedigrees.Clin Genet 2011; 79:273-281.
19. Bennett RL, Steinhaus KA, Uhrich SB, O'Sullivan C, Resta RG, Doyle DL. Recommendations for standardized pedigree nomenclature. Am J Hum Genet 1995; 56:745–752.
20. Sambrook J and Russell WD. Molecular Cloning: A Laboratory Manual. [Internet]. 3rd ed. Cold Spring Harbor Laboratory Press ISBN; 2001. p. 978-087969577-4. Availble from: http://nhjy.hzau.edu.cn/kech/jycz/jczs/ml-introduction/content.pdf.
21. Rozen S, Skaletsky H. Primer3 on the www for general users and for biologist programmers. [internet]. Methods MolBiol 2000; 132:365-368. Availble from: http://www.flyrnai.org/supplement/MMB-RosenSkaletsky.pdf.
22. Rozen S, Skaletsky H. Methods in Molecular Biology, vol.132: Bioinformatics Methods and Protocols. Totowa NJ: S Misener, Kawartz SA; Humana Press Inc; 2000.
23. Azeem Z, Jelani M, Naz G, Tariq M, Wasif N, Kamran-Ul-Hassan Naqvi S, *et al* . Novel mutations in G protein coupled receptor gene *(P2RY5)* in families with autosomal recessive hypotrichosis (LAH3). Hum Genet 2008; 123:515-519.
24. Ali G, Chishti MS, Raza SI, John P, Ahmad W. A mutation in the lipase H *(LIPH)* gene underlie autosomal recessive hypotrichosis. Hum Genet 2007; 121:319-325.
25. Dadkhah E, Ziaee M, Davari MH, Kazemi T, Abbaszadegan MR. Informative STR Markers for Marfan Syndrome in Birjand, Iran. Iran J Basic Med Sci 2012; 15:1020-1025.
26. Khorram-Khorshid HR, Manoochehri M, Nasehi L, Ohadi M, Rahgozar M, Kamali R.Ccr2-64i and Ccr5 Δ32 polymorphisms in patients with late-onset alzheimer's disease; A study from Iran (Ccr2-64i And Ccr5 Δ32 Polymorphisms in Alzheimer's disease). Iran J Basic Med Sci 2012; 15:937-944.
27. Wali A, Ali G, John P, Lee K, Chishti MS, Leal SM, *et al.* Mapping of a Gene for Alopecia with Mental Retardation Syndrome (APMR3) on Chromosome 18q11.2-q12.2. Ann Hum Genet 2007; 71:570–577.
28. Herzog H, Darby K, Hort YJ, Shine J. Intron 17 of the human retinoblastoma susceptibility gene encodes an actively tran-scribed G protein-coupled receptor gene. Genome Res 1996; 6:858–861.