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Gasdermin A Single Nucleotide Polymorphisms and Alopecia Susceptibility in Punjabi Population from North-West India

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Authors' contributions

This work was carried out in collaboration between both authors. Author PPB conceptualized the study and author SR carried out the Lab work. Both the authors contributing equally to the article. Both authors read and approved the final manuscript.

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ABSTRACT

Human Gasdermin A (*GSDMA),* a member of gasdermin gene family, is mainly expressed in skin and stomach. Mutations in its mouse counterpart *Gsdma3*, were found to cause skin diseases characterized by hair loss/ alopecia. As human and mice genes share 75% sequence similarity, present study was designed to check whether natural variability in human *GSDMA* gene was associated with alopecia. Blood samples of 100 alopecia patients and 100 age matched controls were collected and genomic DNA Isolated. All the samples were genotyped for two *GSDMA* SNPs, rs7212938 (V128L) and rs200722398 (V253I) for distribution of alleles along with haplotype analysis. Out of the T and G allele of rs7212938, the G allele count was found to be significantly increased (0.29 to 0.39) among alopecia patients and out of G/A alleles at rs200722398, allele A count was found to be significantly increased (0.06 to 0.13) among alopecia patients. Further haplotype analysis revealed that haplotype combination TGTAGG of rs7212938 and rs200722398 enhanced the susceptibility to alopecia significantly among Punjabi men. Studies on large population sample, other interacting genes and mechanism underlying the observed enhanced susceptibility are required to delineate the role of the observed association between *GSDMA* alleles and relative risk of alopecia.

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Keywords: Gasdermin A; single nucleotide polymorphism; alopecia; haplotype.

1. INTRODUCTION

Gasdermin superfamily is a gene group consisting of gasdermin family genes (*GSDMA, GSDMB, GSDMC, GSDMD*) and gasdermin related genes (*DFNA5/GSDME and DFNB59*). The human gasdermin family shares, 45% overall sequence homology [1]. All Gasdermins except *DFNB59* show similarity in protein structure which consists of two domains, gasdermin N-terminal (NT) and gasdermin C-terminal (CT) that are connected by an unstructured loop. The positions of these terminals are well conserved among the *gsdm* (mice) and *GSDM* (human) gene family. *GSDMA* was the first GSDM family member to be identified in Rim3 mutant mice as a gene positioned near to Rim3 mutation that was causing abnormal skin and hair development in the animals [2].

Human GSDMA protein consists of 445 amino acids [3]. The NT of GSDMA protein causes pore formation when it is cleaved by inflammatory caspase, into N (1 to 251 amino acids) and C (252 to 445 amino acids) terminals [4,5]. NT oligomerizes by binding to the cardiolipin proteins and creates pores in plasma membrane leading to pyroptotic cell death [6,7].

The human genome has one group A gene (*GSDMA* also known by the aliases GSDM, GSDM1 or FKSG9) on chromosome 17q21 and mouse has three orthologues: *Gsdma1*, *Gsdma2* and *Gsdma3* on chromosome 11 [8]. Phylogenetic analysis of the Gsdma cluster shows that human *GSDMA* has 87%,74%, and 73% amino acid sequence similarity with mouse *Gsdma, Gsdma2, and Gsdma3,* respectively; indicating that human *GSDMA* is a counterpart of mouse *Gsdma* [9]. The *Gsdm/GSDM* family genes have been found to be differentially expressed in the epithelium from skin to gastrointestinal tract, in a highly tissue-specific manner [10]. Human *GSDMA* is mainly expressed in skin and stomach [10, 11].

Spontaneous and chemically induced mutations in Mouse *Gsdma3* were found to result in hyperkeratosis and hair loss phenotype in the skin of mice [12]. A study in mice in 2012 [13] brought out the fact that immune mediated destruction of bulge stem cells play crucial role in alopecia.

The mechanism underlying alopecia with severe chronic inflammation observed in the skin of mutant mice [9], was explained in 2015 by Lin and coworkers [4]. They confirmed the presence of nine mutant alleles in the C terminal domain of *gsdma3,* which can lead to gain of function by the protein. These nine mutations were 259 RDW (insertion after residue 259 with mistranslated RDW sequence), T278P, L343P,Y344C, Y344H, A348T, I359N, premature stop at 366, and duplication of E411A412 (412EA).The gain of function was in the form of unmasking of functional NT of the protein, which became free to aggregate at the cell membrane and caused pore formation leading to inflammation, as these mutations were found to disrupt the co-immuno-precipitation between the gasdermin-N and -C domains of *Gsdma3* [5]. Thus, the alopecia-causing Gsdma3 mutants loose the auto-inhibitory interaction between NT and CT, making NT constitutively active and triggering pyroptosis. and become constitutively active in triggering pyroptosis.

Both Human and mouse Gasdermin A genes were observed to be conserved [12,14] and both undergo pyroptosis pathway. As per sequence alignment and 3D model prediction study [3], both the genes (Human and mouse counterpart) adopt two domain structure (N and C terminal domains). Although the particular caspase that cleaves human GSDMA is still unknown, but the pyroptosis and cleavage of N from C terminal was observed to be similar in both mice and humans [3]. Ding et al. [3], established the crystal structure of mice Gsdma3 and all the other gasdermins by sequence alignment in 2016. GSDMA is made up of 14 β sheets and 12 α Helices as shown in table 1. N-terminal contains four α helices and eleven β-sheets. The α-helices are flanked by β-sheets. Helix α4 of NT extends into a long loop which is embedded into the CT leading to inhibition of NT by CT. With the help of three short stranded β-sheets, C domain adopts a compact globular structure. The α1 helix and β1–β2 hairpins act as a primary surface for binding between N and C terminals. Table 1 showing the position of residues in α helices and β sheets.

Table 1. Amino acid position in GSDMA Protein secondary structure [3]

α Helices		β- Sheets	
Helix No	**Residue No**	**Sheet No**	**Residue No**
α 1	1-15	β1	36-41
α 2	122-130	β2	53-55
α 3	138-146	β3	85-94
α 4	179-183	β4	99-102
α 5	257-273	β5	112-120
α 6	276-291	β6	151-159
α 7	293-309	β7	163-167
α 8	326-341	β8	194-197
α 9	344-356	β9	203-209
α 10	358-373	β10	211-214
α 11	394-407	β11	216-219
α 12	425-442	β12	380-383
		β13	410-412
		β14	417-420

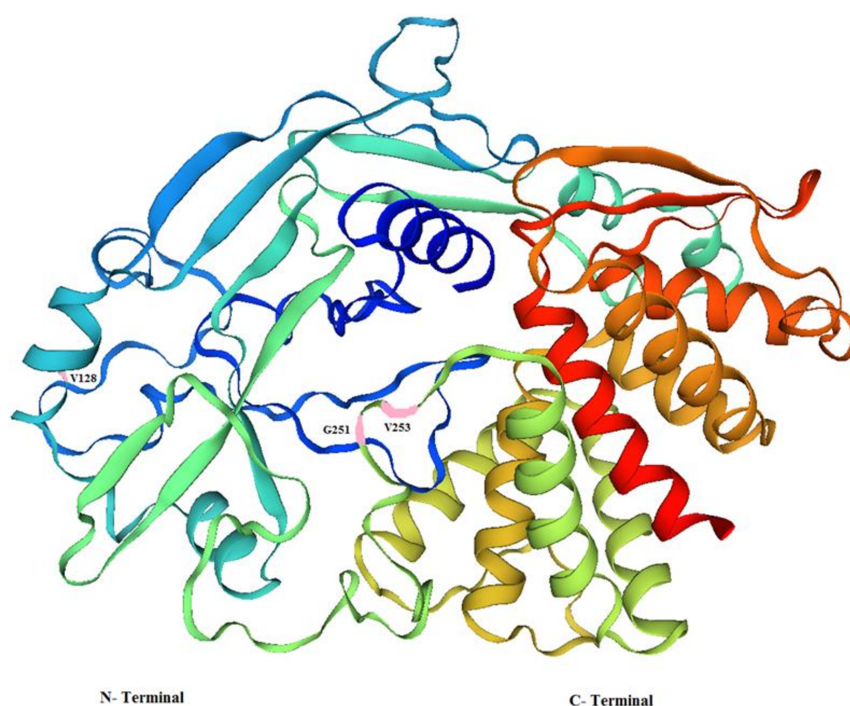

Fig. 1. 3-D Structure of GSDMA protein. Both the SNPs and the tentative caspase cleavage site are highlighted with pink color

Polymorphisms in N and C terminal residues of GSDMA may cause either alteration in pore forming activity or may influence the auto-inhibitory function of the two terminals of protein. So, for the present study two nsSNPs, one from N terminal and one from C terminal were selected to check the variation at population level. rs7212938 (V128L) lies in α 2 helix of N terminal and rs200722398 (V253I) lies near the caspase cleavage site which is at 251th amino acid in C terminal.

2. MATERIALS AND METHODS

2.1 Selection of ns SNP

The presence of nsSNPs in the protein were checked using NCBI dbSNP (Database of Single

Nucleotide Polymorphism). SNPs information like SNP ID, amino acid change and position of *GSDMA* gene were retrieved and subjected to *insilico* analysis using SIFT, PolyPhen, PROVEAN, Mupro, I-Mutant and SNP & GO. Subsequent to this analysis two ns SNPs rs7212938 (V128L) and rs200722398 (V253I) were selected for analysis in Population from Punjab as no such data for the same was available in any database.

2.2 Genotyping of Selected Variants

Intravenous blood (5 mL) sample was collected from 100 alopecia and 100 normal subjects from different regions of Punjab in EDTA tubes with the informed consent of patients. This study has been approved by Institutional clinical ethical committee (IEC No 69 Dated-16-08-2016). Healthy Men of Age: 20 - 80 Years with alopecia and without alopecia were included in the study. Patients reporting any kind of skin disease like Secondary syphilis, Thyroid disease, Iron deficiency, Seborrheic dermatitis, *Lichen planus* and discoid lupus erythematosus and cancer were excluded. Genomic DNA was isolated using standard salting out method [15] and checked for quality on 1% agarose gel.

2.3 Qualitative Analysis of Genomic DNA

The quality of DNA was checked using 1% Agarose gel electrophoresis. Double-stranded DNA fragments naturally behave as long rods, so their migration through the gel is relative to their size. Sharp single bands in the loading wells show the genomic DNA quality as intact high molecular weight DNA. No sample was found to be sheared (Fig. 2).

The genotyping of selected variants was carried out using polymerase chain reaction (PCR)-restriction fragment length polymorphism (PCR-RFLP). The Primer pairs used for the genotyping of these SNPs were designed using Primer blast, GENERUNNER version 3.05 (Hastings software Inc.) and Primer stats. These were synthesized by Integrated DNA Technologies, USA. Details of Primer pairs, PCR optimization conditions are given in table 2. PCR reactions were standardized using 25mM $Mgcl_2$, 10mM dNTPs, 50 pmoles/µl primers, 100 ng/µl genomic DNA and 5 units/ µl of Taq DNA polymerase. Restriction enzymes used for restriction digestion, restriction sites and the incubation conditions are listed in table 3.

2.4 Banding Pattern Analysis of PCR Product by Polyacrylamide Gel Electrophoresis (PAGE)

Fragment separation through polyacrylamide gels was used to separate, identify and purify nucleic acids. Polyacrylamide gels were prepared by chemical co-polymerization of acrylamide monomers with a cross-linking reagent, N,N'-methylene-bisacrylamide in the presence of ammonium persulfate as catalyst; with tertiary amino groups, N,N,N',N'-tetra-methyl-ethylene-diamine (TEMED), provided as accelerators [16]. Fragment size as predicted from the SNPdb gene sequence, were observed on the 10% PAGE followed by banding pattern analysis by Silver staining [17].

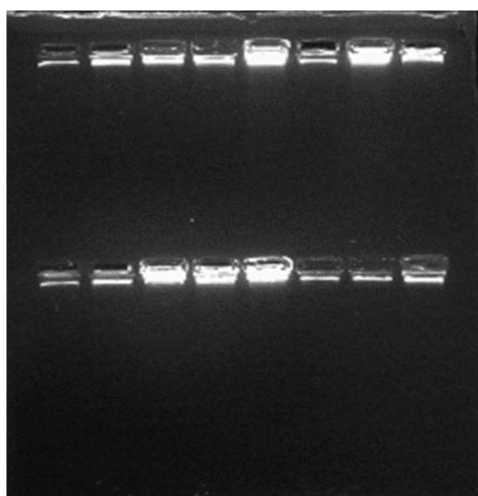

Fig. 2. 1% Agarose Gel showing Genomic DNA bands in wells

Table 2. Details of selected variants genotying with amplification conditions

S.No	Gene	SNP	Primer sequence	PCR Conditions	Methodology used
1.	*GSDMA*	rs7212938	5' GAAGGTGAAGGGAACGGCA 3' (19bp) 5'CAAAGGTCAGTGCAAGGGTCC3' (21bp)	Initial denaturation at 95ºC (5min) Total of 30 cycles 30sec at 95ºC 30sec at 56ºC 1 min at 72ºC Final elongation for 10 min at 72ºC	PCR-RFLP (203bp) (86+117 bp)
2.	*GSDMA*	rs200722398	5'TAAAGGGGGCTGAGTGTAGGAG3'(22bp) 5'ATCTTCACCAAAACATGCCTTCT3' (23bp)	Initial denaturation at 95ºC (5min) Total of 30 cycles 30 sec at 95ºC 30 sec at 57ºC 1min at 72ºC Final elongation for 10 min at 72ºC	PCR-RFLP (306bp) (103+203 bp)

Table 3. Details of restriction conditions and expected restriction patterns ofvariants

Variant	Restriction enzyme	Restriction Site	Restriction digestion mixture (30 µl)	Incubation conditions
rs7212938	HpyCH4III (Thermo Scientific)	5'...ACN▼GT...3' 3'...TG▲NCA...5'	PCR Product-10µl (0.5µg) Buffer-3µl (10x) RE- 1µl (10 U) Sterile water-16 µl	65^{0}C/16 h
rs200722398	RsaI (NEB)	5'...GT▼AC...3' 3'...CA▲TG...5'	PCR Product-10µl (0.5µg) Buffer-3µl (10x) RE- 1µl (10 U) Sterile water-16 µl	37^{0}C/16 h

3. RESULTS

Both validated and non-validated SNPs are present in NCBI dbSNP database. For present study the inclusion criterion used only SNPs validated by 1000 Genome database for further in silico analysis. Out of all variations, only 1000 genome validated nsSNPs with MAF ≥ 0.0001 were selected for the present study and other SNPs which either have MAF < 0.0001 or not validated by 1000 Genome database were excluded from analysis.

On the basis of *in-silico* analysis, out of all two SNPs rs7212938 [GTG ⇒ TTG (V128L)] and rs200722398 [GTA ⇒ ATA (V253I)] were selected for wet lab analysis. The nsSNP rs7212938 is present in N terminal of GSDMA protein at position 128. The nsSNP rs200722398 is present at position 253 in C-terminal, near caspase cleavage site at 251.

3.1 The rs7212938 and rs200722398 SNP Analysis

For rs7212938, the amplified product of 203 bp was obtained, which was subjected to restriction digestion with HpyCH4III (Thermo Scientific) restriction enzyme. The representative 10% PAGE of PCR and restriction digestion products are shown in figure 3**.** Sequences of rs7212938 SNP has been submitted to NCBI SNP db vide accession numbers ss3943621967.

For rs200722398, the amplified product of 306 bp was obtained, which was subjected to restriction digestion with RsaI (NEB). The representative 10% PAGE of PCR and restriction digestion products are shown in Figure 4. Sequence for rs200722398 has been submitted at NCBI SNP db vide accession numbers ss2137544068.

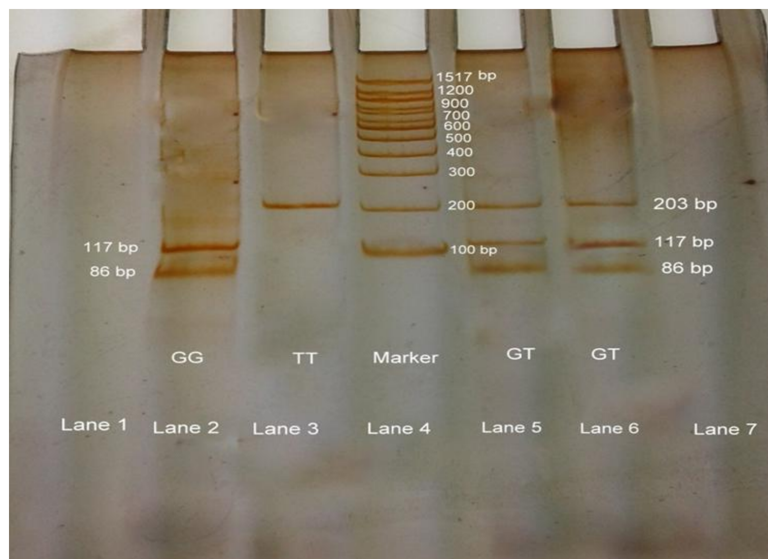

Fig. 3. 10% PAGE showing Genotyping after digestion of 203 bp PCR product of rs7212938 of *GSDMA* gene with HpyCH4III Restriction Enzyme. Lane 2 shows homozygote GG, Lane 3 shows homozygote TT, Lane 5 and 6 shows heterozygote's GT genotypes, Lane 4 shows 100bp DNA Ladder (NEB)

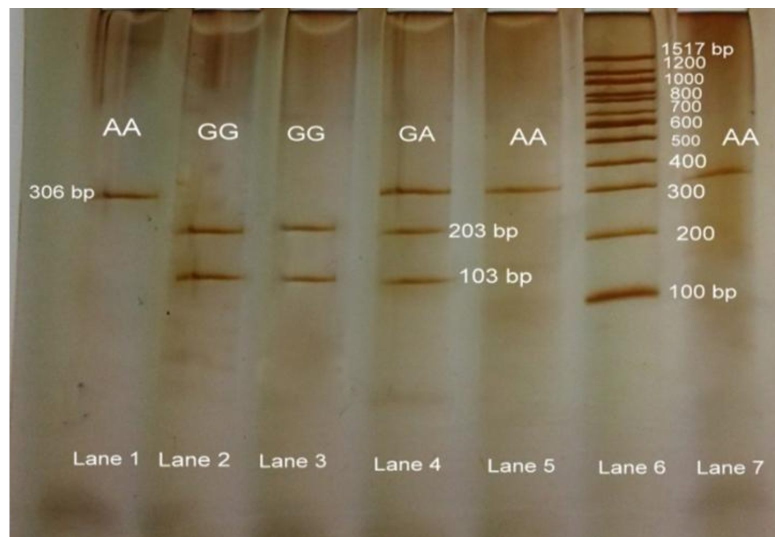

Fig. 4. Genotyping of rs200722398 of *GSDMA* gene using RsaI Restriction Enzyme. Lane 1 shows single band of 306 bp the AA genotype, Lane 2 and 3 show 2 bands with GG genotype, Lane 4 shows 3 bands of GA genotype. Lane 6 shows 100 bp NEB DNA Ladder

Table 4. Worldwide distribution of *GSDMA* gene rs7212938

Population	Alleles	
	T	G
HAPMAP-HCB (Asian)	0.6279	0.3720
HAPMAP-JPT (Asian)	0.5581	0.4418
HAPMAP-CHB (Asian)	0.5853	0.4146
HAPMAP-TSI (Asian)	0.6022	0.3977
HAPMAP-CEU(European)	0.508	0.491
Sub-Saharan African	0.50	0.50
Punjabi Population*	0.71	0.29
Punjabi Alopecia*	0.61	0.39

https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?do_not_redirect&rs=rs7212938
** Present Study*

3.1.1 *GSDMA* rs7212938 variability in Punjabi population

On comparison to population data worldwide, the distribution of T and G alleles at this SNP in population from Punjab is close to other Asian populations as listed in Table 4.

3.1.2 *GSDMA* rs7212938 variability among alopecia patients in Punjabi population

The Allelic distribution of rs7212938 among Normal and Alopecia patients is shown in Table5. The results depict the frequency of T allele (Leu) to be 0.71 and the frequency of G allele (Val) is to be 0.29 amongst Non-effected men. Frequency of T allele among Alopecia patients was found to be decreased (0.61) as compare to Normal and the frequency of G allele was found to be increased (0.39) as compared to Normal subjects. The difference was found to be statistically significant using chi square test (*P* value .0280<.05) and relative risk was found to be 1.174 folds.

3.1.3 *GSDMA* rs200722398 variability among Punjabi population

Apart from present study, genetic polymorphism at the locus rs200722398 has been reported only among other Indian populations, Bengalis from Bangladesh and the Punjabi population from Pakistan. All other populations of the world have reported the locus to be monomorphic. The Indian populations shows two alleles G and A at the locus. The G and A allele frequencies of Punjabi population from present study are different from those observed in other Indian populations as listed in the NCBI SNPdb shown in Table no 6. The distribution of alleles at this

SNP in population of Punjab is closer to Bengalis from Bangladesh, Gujrati Indians from Houston, Texas, Indian Telugu from the UK and Punjabi from Lahore, Pakistan populations as reported at the NCBI site.

3.1.4 *GSDMA* rs200722398 [GTA ⇒ ATA (V253I)] variability among Alopecia patients in Punjabi population

The Allelic distribution frequency among Normal and Alopecia patients is shown in table 7. The results depict the frequency of G allele to be 0.94 and the frequency of A allele is to be 0.06 amongst Normal population. Frequency of G allele in Alopecia patients found to be decreased (0.87) as compare to Normal and the frequency of A allele is found to be increased (0.13) as compared to Normal subjects. The difference in frequencies was found to be statistically significant using chi square test (*P* value .0407<0.05) with relative risk 1.069 fold.

3.2 Haplotype Analysis

In case of *GSDMA* gene variants, TGTAGG haplotype combination on comparison amongst the alopecia patients and normal subjects was found to show statistically significant difference in occurrence. In literature, there are no studies reporting these haplotypes. Table 8 shows the haplotype combinations with relative risk, odd ratio and *P*- value for rs7212938 and rs200722398.

The TGTAGG haplotype combination of *GSDMA* rs7212938 and rs200722398 polymorphism was significantly associated with alopecia risk in Indian Punjabi population. Further studies with larger sample size are required to confirm the results.

Table 5. Comparison of genotypic and allelic disrtibution of rs7212938 between Normal and Alopecia subjects

Sample Group	Genotypes			Alleles		χ^2 (df=1)	*P*-value (S/NS)	Relative Risk	Odds Ratio
	TT	GG	TG	T Count (Freq.)	G Count (Freq.)				
Normal Subjects n=100 (%)	46 (46.00)	4 (4.00)	50 (50.00)	142 (0.71)	58 (0.29)	7.153	.0280 (S)	1.174 (1.018 to 1.358)	1.598 (1.045 to 2.418)
Alopecia Subjects n=100 (%)	28 (28.00)	7 (7.00)	65 (65.00)	121 (0.61)	79 (0.39)				

Table 6. Worldwide distribution of *GSDMA* gene rs200722398

Population	Ref Allele	Alt Allele
GIH (Gujrati Indian from Houston, Texas)	G=0.9951	A=0.0049
ITU (Indian Telugu from the UK)	G=0.9902	A=0.0098
BEB (Bengali from Bangladesh)	G=0.9884	A=0.0116
PJL (Punjabi from Lahore, Pakistan)	G=0.9948	A=0.0052
Punjabi Population*	G = 0.94	A = 0.06
Punjabi Alopecia*	G= 0.87	A = 0.13

https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/
** Present Study*

Table 7. Comparison of genotypic and allelic disrtibution of rs200722398 between Normal and Alopecia subjects

Sample Group	Genotypes			Alleles		x^2 (df=1)	*P*-value (S/NS)	Relative Risk	Odds Ratio
	GG	AA	GA	G Count (Freq.)	A Count (Freq.)				
Normal Subjects n=100 (%)	89 (89.00)	2 (2.00)	9 (9.00)	187 (0.94)	13 (0.06)	4.187	.0407 (S)	1.069 (1.003 to 1.145)	2.055 (1 to 4.289)
Alopecia Subjects n=100 (%)	80 (80.00)	5 (5.00)	15 (15.00)	175 (0.87)	25 (0.13)				

Table 8. Distribution of *GSDMA* gene variants haplotypes among normal and alopecia subjects

Haplotypes	Normal Subjects 2n=200 (Freq)	Alopecia Patients 2n=200 (Freq)	x^2 (df=1)	*P*-value (S/NS)	Relative Risk	Odds Ratio
TG	131 (0.66)	100 (0.5)	Reference			
TA	11 (0.05)	22 (0.11)	6.348	.0118 (S)	1.125 (1.027 to 1.253)	2.62 (1.189 to 5.883)
GG	56 (0.28)	75 (0.38)	6.525	.0106 (S)	1.226 (1.048 to 1.443)	1.754 (1.136 to 2.675)
GA	2 (0.01)	3 (0.01)	0.5556	.4560 (NS)	1.015(0.9713 to 1.075)	1.965(0.394 to 11.21)

4. DISCUSSION

Genetic polymorphism at the locus rs7212938 has been reported from other populations of the world as well and genetic polymorphism at the locus rs200722398 has been reported only among the Indian populations and few other populations from the Asian region. All other populations of the world have reported this locus to be monomorphic.

rs7212938 is a natural variant in dbSNP database and was also found to be associated with asthma in literature. One study in Korean Population claimed that haplotype combination of *GSDMA* and *GSDMB* SNPs (GG (Val) of *GSDMA* rs7212938 and TT of *GSDMB* rs7216389) , both located at 17q21.2 were found to be strongly associated with childhood asthma susceptibility [18]. Both *GSDMA* and *GSDMB* have been linked to asthma pointing to their close gene location and likely coupled regulation and co-expression. Another mechanistic study also linked *GSDMA* and *GSDMB* to asthma as the causative factor of pyroptotic death of airway epithelial cells [19]. The present study also found an increase in G allele amongst alopecia patients.

In a study of all cardiolipin binding proteins, the Cardiolipin binding amino acid propensities were analyzed for these proteins [20]. The study concluded that the hydrophobic amino acids Leu, Ile and Val dominate in cardiolipin binding proteins, constituting 31-40% of residues in such proteins. In a study of sheep retroviral envelop proteins a specific position 501 in the envelop protein ENV was found to be conserved evolutionarily and occupied by Leu, Val, and Ile residues. However a later study by Cote et al (2012) [21] brought out the fact that the Leu-Val difference in these closely related viruses does lead to difference in the pH-dependent membrane fusion and cell entry.

In GSDMA, V to L 128 is within the conserved alpha 2 fold of GSDMA NT of the protein. Interestingly in mouse Gsdma3, human GSDMD, GSDMB and DFNA5 all have Leu at the corresponding position, thus pointing to likely better or equal binding efficiency and or membrane aggregation during pore formation. As

pointed in case of viral envelop proteins, the pore forming property may be influenced by this change due to smaller side chain of valine as compared to leucine.

Conceptually the pore formation by NT involves three steps (i) interfacing with membrane for binding (ii) formation of secondary Beta pleated sheet structure (iii) insertion of Beta sheets to form the barrel structure of the pore [22]. Due to its larger side chains Leu shows higher hydrophobicity as compared to Val with a small side chain. The decreased frequency of Val amongst alopecia patients may influence the mechanistics of the beta barrel formation. Each of the pore structure of Gsdma3 was found to be constituted of 27 subunits [3]. Thus as pointed by Cote et al. [21] viral study, the cumulative effect of 27 units of Val containing NT are hypothesized to influence pore formation. However the pores from the two type of variants need to be studied thermodynamically in vitro as well as in vivo to reach a conclusion.

The rs200722398 [GTA ⇒ ATA (V253I)] lies between β11 sheet and α5 helix of C terminal near the caspase cleavage site. The site is very near the Caspase cleavage site as observed in other gasdermin proteins, thus may influence the cleavage and production of NT responsible for inflammatory pyroptosis. Presently no caspase has been identified for cleavage of GSDMA in humans, thus no conclusion as to the deleterious or otherwise effect of this SNP can be drawn.

5. CONCLUSION

Alopecia is an inflammatory disease and in mice its association with pyroptosis gave the hint of association between alopecia and pyroptosis in humans also. The SNPs analyses along with their Haplotype combinations have been observed to display statistically significant association with susceptibility to alopecia, though no mechanistic explanation is forthcoming at present. Further study on large population sample along with other interacting genes of pyroptosis pathway are needed to reach at definite conclusion.

CONSENT TO PARTICIPATE AND ETHICAL APPROVAL

This study was approved by the Institutional Clinical ethical Committee of Punjabi University, Patiala, Punjab, India (IEC No-69 dated 16-08-2016). All the subjects gave a written informed consent with a signature or thumb impression.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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