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Association of *ISL1* polymorphisms and eosinophilic levels among otitis media patients

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Abstract

Background: Otitis media (OM) is a middle ear inflammatory complex disorder involving genetic and environmental factors. It onsets during childhood and often recurs and perplexes in genetically susceptible patients. Previously, murine models had shown the association of *ISL LIM* homeobox 1 (*ISL1*) gene with otitis media with effusion.

Aim: To investigate the association of *ISL1* genetic variants with otitis media.

Subjects and methods: A total of 285 cases and 277 controls were recruited for the study. The entire coding region of *ISL1* gene was genotyped using Sanger sequencing or single-strand conformation polymorphism methods. Genotype, haplotype, *in silico* analysis, and linkage disequilibrium analysis were performed.

Results: The variants rs2303751 (c.504A>G) and rs121913540 (c.513G>A) were associated with OM, and the OR (95%CI) was 0.74 (0.57–0.95) and 0.43 (0.20–0.91), respectively. Besides, the rs2303751 AA genotype was associated with elevated eosinophil numbers in OM when compared to controls. The 5 SNP haplotype analysis of SNPs c.-492A>G, c.504A>G, c.513G>A, c.576C>T, and c.*651A>T revealed A-A-G-C-A to be a risk haplotype in females whereas the 3 SNP haplotype analysis of SNPs c.504A>G, c.513G>A, and c.567C>T suggested G-A-C as protective and A-G-C to be a risk haplotype for otitis media.

Conclusion: Ours is the first report which shows a significant association of *ISL1* variants (rs2303751 and rs121913540) with hearing-related disorder like otitis media in humans. These results implicate the possible role of *ISL1* gene in the etiopathology of otitis media. The replication of the study in other ethnic populations may strengthen our findings.

KEYWORDS
genetic association, haplotype, *ISL1*, otitis media, single-nucleotide polymorphism

1 | INTRODUCTION

Otitis media (OM), the middle ear malady, is considered a multifactorial complex disorder backed by host genetic, epigenetic, immunogenetic, pathogenic (viral or bacterial), and accessory environmental factors. The Eustachian tube dysfunction and frequent upper respiratory tract infections also contribute to the pathology of the disease. It is one of the most underrated

otological condition which is often overlooked until it ramifies into the chronic stage like chronic otitis media with effusion (COME) and chronic suppurative otitis media (CSOM). The global prevalence of hearing loss associated with OM was estimated to be 30 per 10,000 but it can vary in low- and middle-income countries. Monasta et al. have shown that globally 31 million new cases of CSOM arise every year.[1,2] Developing countries like China had reported the prevalence of CSOM to be 9.82%.[3] A Brazilian study has reported about 90% of the patients with intracranial complications, due to OM, suffered hearing loss and 7.8% succumbed to death.[4] Third world countries like India face a huge burden due to OM and its secondary complications. Toward the Indian context, the prevalence studies on OM are scattered and the prevalence ranges from 5.2%–15.3%.[5–7] However, molecular genetic studies on OM in the Indian population are scarce.

Erstwhile, studies on the genetics of OM had showed the ethnic variation in prevalence and the disease strongly implies to a specific ethnic population.[8,9] Many studies had shown the association of OM, leaned toward the genetic risk aspects.[10,11] The heritability study on twins and triplicates revealed a strong association between the number of episodes and genetic makeup of the individual.[12] OM susceptibility with chromosomal anomalies was also reported.[13–15] From all the previous studies worldwide, the fact is now well established that host genetics plays a crucial role during the progress of disease pathophysiology. There is a strong genetic component which is not unitary but distributed differently across the varied phenotypes and their different pathogenic stages.[16]

Taking into account the practical limitations of working on human populations and human samples, an array of murine models had been devised for many disorders. Similarly, there is a wide range of studies on transgenic or knockout mice that tried to suss out the genes and genetic variations that confer susceptibility to OM in mice. The animal model studies on *EYA* transcriptional coactivator and phosphatase 4 (Eya4), ectopic viral integration site 1 gene *(Evi1)*, TGFβ-induced factor homeobox 1 *(TGIF1)*, Nischarin *(Nisch)*, and F-box protein 11 *(FBXO11)* loci have illustrated a potential risk areas for OM in humans.[17–21]

The identification of missense mutation of the *ISL* LIM homeobox 1 (*ISL1*) gene and its association with low penetrance otitis media in a murine model[22] are the major drivers for the present study. We tried to extrapolate the genetic association study of the reported *ISL1* gene into a case–control module. Our investigation is focused on chronic phenotypes of OM (CSOM) which may be caused due to the veiled genetic factor(s).

ISL LIM homeobox 1 (ISL LIM homeobox 1 also known as Insulin gene enhancer protein 1 or Islet-1) is a protein-coding gene that encodes LIM/homeodomain family of transcription factors. The translated product of *ISL1* binds to the enhancer region of the Insulin gene and plays a regulatory role. Unlike the murine that have a single isoform of *ISL1* in their chromosome 1, the humans have seven isoforms in chromosome 5.[22] Role of *ISL1* contextual to hearing disorders like OM in humans has not been reported until now. Nonetheless, *ISL1* expression has shown in many mammalian tissues and cell lines. Therefore, the present study was undertaken to scrutinize the entire coding region (six exons) of *ISL1* along with their exon–intron boundaries for the association of genetic variants responsible for OM susceptibility.

2 | MATERIALS AND METHODS

2.1 | Study subjects

The study included patients with OM (*N* = 285) and healthy controls (*N* = 277). The patients with OM were recruited from the Department of Ear, Nose, and Throat, SCB medical college, Cuttack and Capital Hospital, Bhubaneswar, Odisha, India. The patient's diagnosis was based on otoscopic examination of tympanic membrane and the middle ear. Otoscopy reveals the status of tympanic membrane in patients which can be bulged or retracted due to the accumulated middle ear effusion and middle ear pressure. An intact tympanic membrane (TM) with middle ear fluid indicates AOM or ROME whereas a perforated TM with purulent discharge from ear indicates CSOM. The hearing loss due to OM was measured using pure tone audiometry, impedance testing, and tympanometry. Following the diagnosis, the patient's history was recorded. The control group was healthy volunteers of same ethnic population willing to participate in the study with no history of otitis media in their family. The subjects with systemic, congenital, and acquired diseases of any other form were excluded. The mean age of cases and control subjects is 32.4 ± 14.4 and 31.3 ± 10.8, respectively. All the subjects signed an informed consent, and Institutional Ethical Committee approved the study protocol.

2.2 | Genotyping

Peripheral venous blood (5 ml) was collected from both cases and controls in K2 EDTA vials, and complete blood count (CBC) was performed using automated CBC counter Sysmax XS-800i (Norderstedt, Germany). DNA was isolated from 4 ml of blood by a rapid non-enzymatic method[23] where the blood cells were lysed, deproteination was done by salting out, and DNA was precipitated by absolute ethanol. The quantity and quality of isolated DNA was analyzed by Nanophotometer (Implen, GmBH, Munchen, Germany). The entire coding region of *ISL1* (NM_002202.3) consisting of 6 exons was amplified by using the primers and conditions described previously.[24] Amplified products of exon 1, 2, 3, 4, and 6 were purified by QIAquick gel extraction kit (Qiagen, Gmbh, Hilden, Germany) following manufacturer instructions. Each purified amplicon was sequenced in forward and reverse direction using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the same primers used for amplification. Obtained sequences were assembled to reference sequence and analyzed using Sequencher v5.4.6 (Gene Codes Corporation, Ann Arbor, MI, USA). The exon 5 region was genotyped using single-strand conformation

polymorphism (SSCP) method where the amplified product was denatured using the 2x SSCP dye.[25] The denatured products were snap cooled and electrophoresed at room temperature. The gel was then silver-stained, and bands were visualized on the gel illuminator. About 25% of samples analyzed by SSCP were sequenced for their concordance.

2.3 | Association analysis

Genotype and allele frequencies were calculated by the direct counting method. We tested all the SNPs for deviation from the Hardy–Weinberg equilibrium in the controls using the HWE calculator.[26] Association between the genotyped SNPs and OM was tested by using Fisher's exact test. Subsequently, haplotype construction and their frequencies among the alleles investigated were calculated using SNP*Alyze* V8.0.2 (Dynacom Co Ltd., Chiba, Japan) based on the EM algorithm and a maximum likelihood approach. The *p*-value <0.05 was considered significant. The linkage disequilibrium (LD) of all possible two-way combinations of SNPs with the absolute value of the correlation coefficient using D' and r^2 was performed using Haploview 4.2 software (https://www.broadinstitute.org/haploview/haploview).

2.4 | *In silico* analysis of *ISL1* sequence variants

The disease-causing potential of the *ISL1* sequence variants was predicted by the open source online tool: Mutation Taster (http://www.mutationtaster.org) which determines the probability for the variations to be either a pathogenic allele or a benign polymorphism.

3 | RESULTS

3.1 | Demographic characteristics

The OM cases recruited in the study comprised 150 males and 135 females whose mean age was 32.9 ± 15.2 and 32 ± 13.6, respectively. Upon diagnosis, 84.6% of cases were found to be CSOM. About 58.2% of the cases had mixed hearing loss, and rest showed conductive hearing loss. From recruited cases, 21% displayed a positive family history. The detailed characteristics are shown in Table 1.

TABLE 1 Clinical characteristics of otitis media cases and controls

Parameters	Cases (*N* = 285)	Controls (*N* = 277)
Sex, *n* (%)		
Males	150 (52.6%)	162 (58.5%)
Females	135 (47.4%)	115 (41.5%)
Age (Mean ± *SD*)		
Males	32.9 ± 15.2	31.4 ± 11.0
Females	32 ± 13.6	31.1 ± 10.5
Type of hearing loss, *n* (%)		
Mixed	166 (58.2%)	NA
Conductive	119 (41.7%)	NA
No. of ears affected, *n* (%)		
Unilateral	126 (43.8%)	NA
Bilateral	159 (55.8%)	NA
Types of OM, *n* (%)		
AOM	06 (02.1%)	NA
CSOM	241(84.6%)	NA
ROME	38 (13.3%)	NA
Family history, *n* (%)	60 (21.0%)	NA

Abreviations: AOM, acute otitis media; CSOM, chronic suppurative otitis media; NA, not applicable; ROME, recurrent otitis media with effusion; SD, standard deviation.

3.2 | Genetic analysis

Genotyping of *ISL1* in cases and controls revealed five known polymorphisms rs3762977 (c.-492A>G), rs2303751 (c.504A>G), rs121913540 (c.513G>A), rs121913541 (c.567C>T), and rs1017 (c.*651A>T). The position of the SNPs is illustrated in Figure 1. All the common variants were found to be in HWE except c.567C>T. The frequency of these variants in the cases and controls for the rare alleles along with their pathogenicity status is shown in Table 2. Association evaluation for the identified SNPs using 3 genetic models (dominant, co-dominant, and recessive) was performed. Testing of these variants identified an allelic association of SNP c.504A>G (p = 0.024) between with OM. The SNP c.513G>A displayed a homozygous "GG" (p = 0.028) association in dominant model, heterozygous "GA" (p = 0.028) association in co-dominant model, and allelic "A" (p = 0.030) association (Table 3). The representative chromatograms for different genotypes of c.504A>G and c.513G>A are shown in Figure 2A. The gender stratified association analysis brought out the gender-related (females) allelic "G" association (p = 0.019) in c.504A>G whereas in dominant model genotype "AA" association (p = 0.043) was found in c.504A>G. The SNP c.513G>A showed a significant allelic "A" (p = 0.028) association. Dominant model revealed "GG" (p = 0.026), and co-dominant model displayed "GA" (p = 0.026) genotypic association with significant frequency difference between cases and controls in females (Table 4). The PCR-SSCP analysis of exon 5 revealed no variations in cases and controls (Figure 2B).

Haplotype analysis for the genotyped SNPs was performed for the 5 SNPs and 3 SNPs in coding region individually (frequency >1%) (Table 5). The haplotype A-A-G-C-A containing the allele "A" of c.-492A>G, "A" of c.504A>G, "G" of c.513G>A, "C" of c.567C>T, and "A" of c.*651A>T, respectively, was found statistically different (p = 0.013) in female cases compared to controls. The analysis of 3 SNPs c.504A>G, c.513G>A, and c.567C>T harbored in the coding region displayed

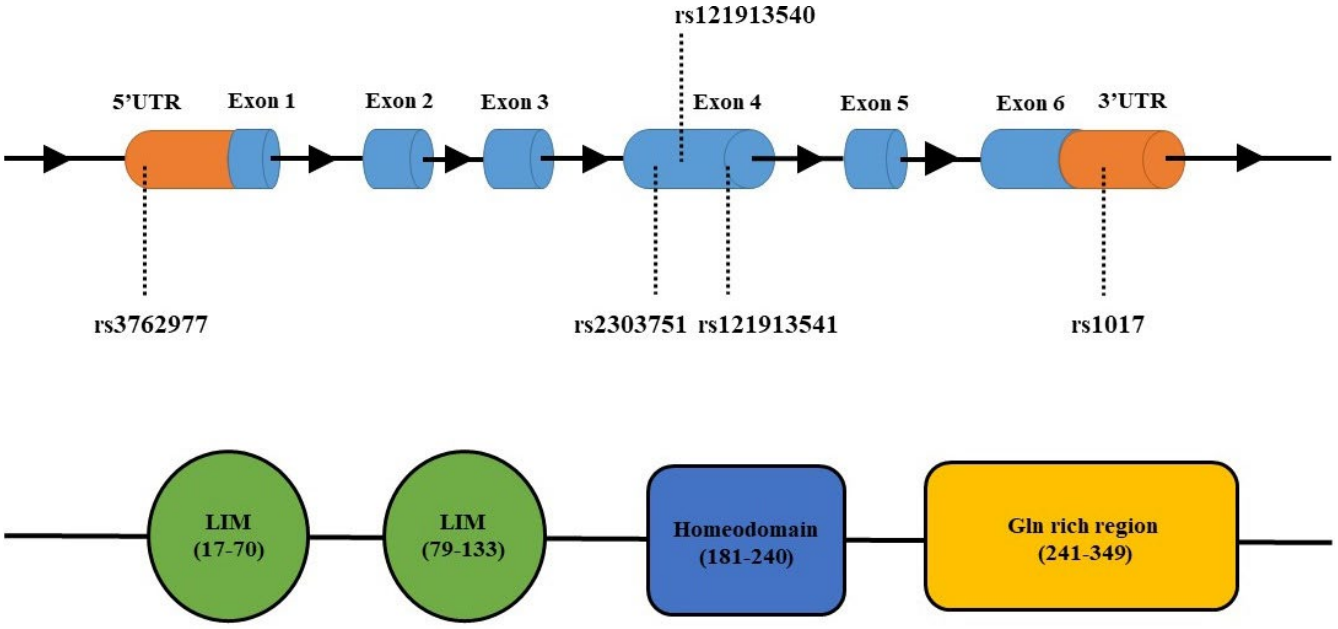

FIGURE 1 Schematic representation of ISL1 (5q11.1) gene and the position of studied variants. ISL1 encodes for a transcription factor containing two LIM domains, one homeodomain and a Glu-rich region

TABLE 2 *ISL LIM* homeobox 1 genetic variants with their pathogenic potential and frequency distribution in otitis media cases and controls

SNP Position	rs number	Position	Pathogenicity	Minor allele frequency		HWE (χ^2 value)	*p*-value
				Cases	Controls		
c.-492A>G	rs3762977	5′UTR	Benign polymorphism	0.14	0.16	3.19	0.07
c.504A>G	rs2303751	P168P	Benign polymorphism	0.28	0.34	3.27	0.07
c.513G>A	rs121913540	S171S	Disease causing	0.02	0.04	0.62	0.43
c.567C>T	rs121913541	N189 N	Disease causing	0.01	0.02	34.0	0.00
c.*651A>T	rs1017	3′UTR	Benign polymorphism	0.32	0.35	0.49	0.48

Note:: Pathogenicity was determined using online tool: www.mutationtaster.org.
Abbreviation: HWE, Hardy–Weinberg equilibrium.

the frequency of G-A-C was more in controls compared to cases (p = 0.014); conversely, A-G-C showed increased frequency in cases (p = 0.034) as observed in overall as well as in females.

The pairwise LD analysis revealed the D', r^2, LOD, and distance for the analyzed SNPs. The LD block for 5 SNPs and 3 SNPs in coding region was constructed (Figure 3). The complete LD was observed between the two SNPs c.504A>G and c.513G>A (D' = 1, r^2 = 0.052) whereas the SNPs c.-492A>G and c.*651A>T showed strong LD (D' = 0.95, r^2 = 0.296).

3.3 | Correlation between Eosinophils and OM

Complete blood count analysis of subjects showed abnormally high eosinophils in 30.25% of the cases. Secondary analysis of these cases revealed eosinophil percentage in blood was significantly associated with c.504A>G polymorphism (p < 0.0001, ANOVA) and was higher in patients with AA genotype than AG and GG genotypes (Figure 4).

4 | DISCUSSION

The present study was conducted to identify the association of *ISL1* gene with OM. *ISL1* as a candidate gene for OM was chosen as per the study on *dearisch* mice.[22] Though *ISL1* is a transcription factor which helps in regulation of insulin, it has also been reported to be involved in many other human disorders.[24,27,28] OM onsets during childhood, it recurs and the clinical symptoms aggravate with increasing episodes and age. The patients recruited in the present study had a mean age of 32.4 ± 14.4, which implies the cases suffered several episodes of OM and the condition perplexed into CSOM. The inflammatory condition in the middle ear often diffuses into the inner ear which may be the reason for increased mixed hearing loss patients (58.2%) in the present

TABLE 3 Genotypic and allelic frequencies of *ISL1* polymorphisms in otitis media cases and controls

SNP position (rs number)	Model	Genotype frequency					Allele frequency				
			Cases	Controls	*p*-value	OR (95% CI)		Cases	Controls	*p*-value	OR (95% CI)
c.-492A>G (rs3762977)	Dominant	AA	75 (0.74)	59 (0.68)	0.338	1.37 (0.73–2.59)	A	174 (0.86)	146 (0.84)	0.564	0.84 (0.48–1.46)
	Co-dominant	AG	24 (0.24)	28 (0.32)	0.252	0.66 (0.34–1.25)	G	28 (0.14)	28 (0.16)		
	Recessive	GG	2 (0.02)	0	0.500	NA					
c.504A>G (rs2303751)	Dominant	AA	153 (0.54)	127 (0.46)	0.064	1.37 (0.99–1.91)	A	412 (0.72)	365 (0.66)	**0.024**	0.74 (0.57–0.95)
	Co-dominant	AG	106 (0.37)	111 (0.40)	0.490	0.89 (0.63–1.25)	G	158 (0.28)	189 (0.34)		
	Recessive	GG	26 (0.09)	39 (0.14)	0.086	0.61 (0.36–1.04)					
c.513G>A (rs121913540)	Dominant	GG	275 (0.96)	255 (0.92)	**0.028**	2.37 (1.10–4.86)	G	560 (0.98)	532 (0.96)	**0.03**	0.43 (0.20–0.91)
	Co-dominant	GA	10 (0.04)	22 (0.08)	**0.028**	0.42 (0.19–0.90)	A	10 (0.02)	22 (0.04)		
	Recessive	AA	0	0	NA	NA					
c.567C>T (rs121913541)	Dominant	CC	279 (0.97)	268 (0.97)	0.445	1.54 (0.58–4.35)	C	563 (0.98)	543 (0.98)	0.349	0.61 (0.24–1.55)
	Co-dominant	CT	5 (0.017)	7 (0.02)	0.572	0.69 (0.24–1.98)	T	7 (0.01)	11 (0.02)		
	Recessive	TT	1 (0.003)	2 (0.01)	0.619	0.48 (0.03–4.19)					
c.*651A>T (rs1017)	Dominant	AA	42 (0.46)	40 (0.41)	0.557	1.22 (0.69–2.16)	A	123 (0.68)	127 (0.65)	0.743	0.91 (0.59–1.39)
	Co-dominant	AT	39 (0.43)	47 (0.48)	0.467	0.79 (0.45–1.39)	T	59 (0.32)	67 (0.35)		
	Recessive	TT	10 (0.11)	10 (0.10)	1.000	1.07 (0.41–2.79)					

Note:: Statistically significant differences were analyzed using Fisher's exact test; bold font presents $p < 0.05$; OR, odds ratio; CI, confidence intervals; NA, not applicable; dominant model (AA vs. Aa+aa); co-dominant model (Aa vs. AA+aa); recessive model (aa vs. AA+Aa).

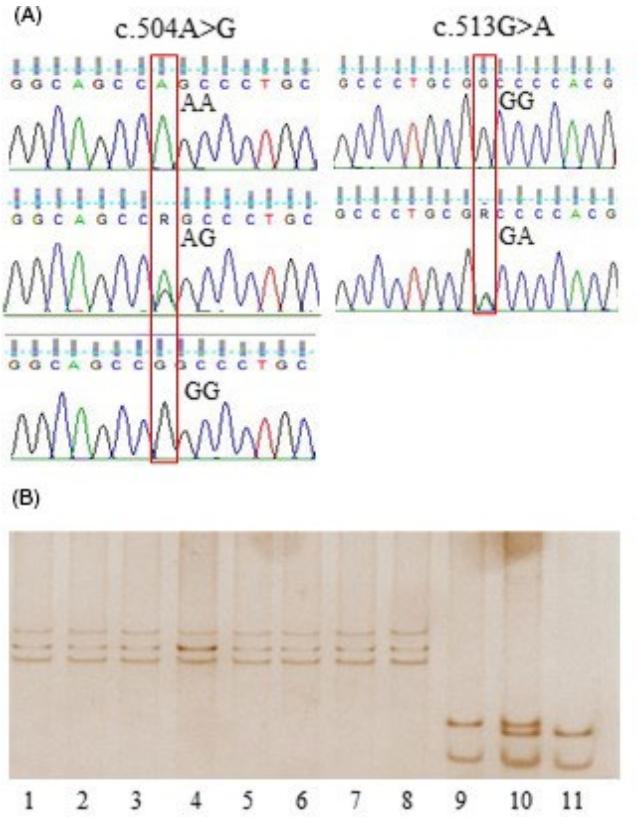

FIGURE 2 (A) Sequencing chromatograms of different genotypes at ISL1 c.504A>G and c.513G>A loci. (B) Single-strand conformation polymorphism (SSCP) silver-stained gel showing no variations in ISL1 exon 5 (lane 1–8). Lane 9–11 is positive controls with different genotypes (of other gene)

study. Previous studies have also shown the association of CSOM with mixed hearing loss.[29] Positive family history was also found in 21% of the cases, indicating the genetic basis of the clinical condition, earlier studies also confirmed that predisposition of developing OM is genetically determined.[12]

Withal our finding shows a significant association of *ISL1* variants c.504A>G and c.513G>A with otitis media. The SNP c.504A>G shows an overall as well as gender-related (female) allelic association. The decreased minor allele "G" frequency in cases (0.28) compared to controls (0.34) and the odds ratio OR=0.741 indicates its protective role in the disease process (Table 4). The variant c.513G>A also displayed an allelic "A" association with OM. In dominant model (GG vs. GA+AA), association of homozygous "GG" and the relatively increased odds ratio (OR) suggests the genotype GG to be a risk genotype. The co-dominant model (GA vs. GG+AA) revealed a weak association of GA with OM and the OR indicates the genotype to be less likely to develop OM. The minor allele frequency of c.513G>A in controls overstood the cases (Tables 3 and 4). In both the SNPs (c.504A>G and c.513G>A), odds of exposure for minor alleles of cases were lower than that of controls hence, indicating a protective role of these alleles against the disease pathology. However, in case of "GG" for c.513G>A, the OR=2.373 and the increased genotypic frequency in cases indicated it as risk genotype, prone to susceptibility for otitis media.

The haplotype analysis for the 5 SNPs c.-492A>G, c.504A>G, c.513G>A, c.576C>T, and c.*651A>T affirmed a significant association of A-A-G-C-A specific to females. The haplotype containing non-protective alleles "A" of c.504A>G and "G" of c.513G>A showed an increase in the frequency in cases, which portends the role of this haplotype to be a risk haplotype in females. The haplotype analysis of 3 SNPs in coding region revealed the haplotype G-A-C containing protective alleles "G" of c.504A>G and "A" of c.513G>A showed increased frequency in controls which further portends the role of this haplotype to be protective. Conversely, the frequency of A-G-C haplotype in overall case–control and in females was found to be significantly increased in cases when compared to controls suggesting it as a risk haplotype in OM (Table 5). The statistically significant *p*-values of these haplotypes assured them to be non-random and also indicated the role of these polymorphisms in the disease pathology to be synergistic rather than solitary. The complete LD between the two SNPs c.504A>G and c.513G>A with D' = 1 might be due to the less physical distance. Even though the SNPs c.-492A>G and c.*651A>T did not show any association with otitis media, a strong LD of D' = 0.95 was found between them.

The allelic, genotypic, and haplotype association, particularly with the females in the present study, may be based on the ability of females to produce much larger inflammatory immune response to tissue damage than males in case of inflammatory diseases.[30] Isl1 had been reported to modulates the expression of transcription factors C/EBP which cooperates with NF-κB for cytokine induced Fas expression; this mechanism contributes to β-cell apoptosis in type 1 diabetes mellitus.[31] Similarly, the associated *ISL1* genetic variants and the haplotypes of females in coalition with other genes may elicit inflammatory response in OM. Isl1 has also been shown to physically interact with both JAK1 and STAT3, forming a complex in both human and monkey immortal cell lines.[32] This results in the activation of STAT3. JAK1 is also activated and can dock to recruit further signaling proteins. STAT3 is necessary for lung and bladder epithelium to respond effectively to gram-negative bacteria.[33,34] Without *ISL1*, the function of these genes in the prevention of infection or inflammation via innate immunity is potentially disrupted. Associated *ISL1* genetic variants in this study in alliance with other genetic variants in humans may contribute predisposition to otitis media by affecting the appurtenance, amount, or evasive nature of middle ear mucosal secretions.

The secondary analysis of ANOVA for eosinophils in whole blood of subjects and the different genotypes of c.504A>G revealed the eosinophil percentage to be higher in "AA" genotype than "AG" and "GG" genotype in cases. This may be due to the protective role of the "G" allele which showed fewer eosinophils in the blood. The middle ear mucosa is known to be from the respiratory origin hence is highly vascularized by the occipital or posterior arteries, branches of the maxillary artery, and other minor arteries. The chance of wandering eosinophils in the systemic circulation to enter the affected middle ear is very high, especially when there are pathogens and negative pressure in the middle ear. The eosinophils once in the middle ear can be detrimental for the auditory orchestra (includes middle and

TABLE 4 Gender-based genotype and allelic frequencies of *ISL1* gene polymorphisms in otitis media cases and controls

Position (rs number)	Model	Genotype Allele	Male				Female			
			Cases	Controls	*p*-value	OR (95%) CI)	Cases	Controls	*p*-value	OR (95%) CI)
c.- 492A>G (rs3762977)	Dominant	AA	40 (0.75)	32 (0.75)	1.000	1.06 (0.41–2.60)	35 (0.73)	27 (0.61)	0.271	1.69 (0.69–3.91)
	Co-dominant	AG	12 (0.22)	11 (0.25)	0.812	0.85 (0.33–2.23)	12 (0.25)	17 (0.38)	0.183	0.53 (0.22–1.32)
	Recessive	GG	1 (0.01)	0	1.000	Inf (0.090–Inf)	1 (0.02)	0	1.000	Inf (0.10–Inf)
		A	92 (0.86)	75 (0.87)	1.000	1.04 (0.45–2.31)	82 (0.85)	71 (0.81)	0.434	0.71 (0.34–1.56)
		G	14 (0.13)	11 (0.13)			14 (0.15)	17 (0.19)		
c.504A>G (rs2303751)	Dominant	AA	79 (0.52)	79 (0.48)	0.499	1.17 (0.76–1.81)	74 (0.55)	48 (0.41)	**0.043**	1.69 (1.03–2.79)
	Co-dominant	AG	55 (0.36)	60 (0.37)	1.000	0.98 (0.61–1.57)	51 (0.38)	51 (0.44)	0.304	0.76 (0.46–1.25)
	Recessive	GG	16 (0.10)	23 (0.14)	0.398	0.72 (0.38–1.38)	10 (0.07)	16 (0.14)	0.101	0.49 (0.22–1.08)
		A	213 (0.71)	218 (0.67)	0.340	0.84 (0.60–1.19)	199 (0.73)	147 (0.64)	**0.019**	0.63 (0.43–0.92)
		G	87 (0.29)	106 (0.33)			71 (0.26)	83 (0.36)		
c.513G>A (rs121913540)	Dominant	GG	142 (0.94)	149 (0.92)	0.375	1.55 (0.61–3.71)	133 (0.98)	106 (0.92)	**0.026**	5.64 (1.43–26.37)
	Co-dominant	GA	8 (0.05)	13 (0.074)	0.375	0.64 (0.26–1.60)	2 (0.01)	9 (0.08)	**0.026**	0.18 (0.04–0.69)
	Recessive	AA	0	0	NA	NA	0	0	1.000	NA
		G	292 (0.97)	311 (0.95)	0.286	0.61 (0.26–1.44)	268 (0.99)	221 (0.96)	**0.028**	0.18 (0.04–0.72)
		A	8 (0.02)	13 (0.04)			2 (0.01)	9 (0.04)		
c.567C>T (rs121913541)	Dominant	CC	145 (0.96)	158 (0.97)	0.742	0.73 (0.22–2.49)	134 (0.99)	110 (0.96)	0.097	6.09 (0.82–72.25)
	Co-dominant	CT	4 (0.02)	2 (0.01)	0.433	2.19 (0.50–11.63)	1 (0.01)	5 (0.04)	0.097	0.16 (0.01–1.22)
	Recessive	TT	1 (0.01)	2 (0.01)	1.000	0.54 (0.04–4.66)	0	0	1.000	NA
		C	294 (0.98)	318 (0.98)	1.000	1.08 (0.34–3.44)	269 (0.99)	225 (0.98)	0.116	0.19 (0.02–1.38)
		T	6 (0.02)	6 (0.02)			1 (0.01)	5 (0.02)		
c.*651A>T (rs1017)	Dominant	AA	15 (0.33)	24 (0.44)	0.305	0.62 (0.27–1.47)	27 (0.58)	16 (0.37)	0.059	2.309 (1.00–5.28)
	Co-dominant	AT	24 (0.53)	26 (0.48)	0.688	1.23 (0.54–2.66)	15 (0.32)	21 (0.48)	0.135	0.51 (0.22–1.18)
	Recessive	TT	6 (0.13)	4 (0.07)	0.505	1.92 (0.53–6.33)	4 (0.08)	6 (0.14)	0.513	0.59 (0.18–2.12)
		A	54 (0.60)	74 (0.68)	0.234	1.45 (0.81–2.61)	69 (0.75)	53 (0.62)	0.075	0.53 (0.29–1.01)
		T	36 (0.40)	34 (0.31)			23 (0.25)	33 (0.38)		

Note:: Statistically significant differences were analyzed using Fisher's exact test; bold font presents $p < 0.05$; CI, confidence intervals; Inf, infinity; NA, not applicable. Dominant model (AA vs. Aa+aa); co-dominant model (Aa vs. AA+aa); recessive model (aa vs. AA+Aa).

TABLE 5 Haplotypes and frequencies for 5 SNPs and 3 SNPs of analyzed *ISL1* variants in otitis media cases and controls

	Overall (*N* = 151)				Male (*N* = 76)				Female (*N* = 75)			
Haplotype[a]	Cases (*N* = 71)	Controls (*N* = 72)	Permuted *p*-value	*p*-value	Cases (*N* = 42)	Controls (*N* = 34)	Permuted *p*-value	*p*-value	Cases (*N* = 37)	Controls (*N* = 38)	Permuted *p*-value	*p*-value
A-A-G-C-A	0.651	0.582	0.205	0.213	0.571	0.616	0.604	0.574	0.743	0.550	7.0E−03	**0.013**
A-G-G-C-T	0.133	0.179	0.295	0.262	0.155	0.205	0.47	0.422	0.108	0.144	0.581	0.505
G-G-G-C-T	0.101	0.125	0.507	0.511	0.107	0.073	0.454	0.476	0.094	0.184	0.142	0.113
A-A-G-C-T	0.051	0.043	0.752	0.756	0.071	0.030	0.369	0.263	0.027	0.055	0.395	0.396
G-G-A-C-T	0.013	0.020	0.627	0.603	0.024	0.029	0.621	0.830	0	0.012	0.976	0.351
	Overall (*N* = 562)				**Male (*N* = 311)**				**Female (*N* = 250)**			
Haplotype[b]	Cases (*N* = 285)	Controls (*N* = 277)	Permuted *p*-value	*p*-value	Cases (*N* = 150)	Controls (*N* = 161)	Permuted *p*-value	*p*-value	Cases (*N* = 135)	Controls (*N* = 115)	Permuted *p*-value	*p*-value
A-G-C	0.717	0.659	0.045	**0.034**	0.671	0.699	0.487	0.451	0.737	0.639	0.018	**0.018**
G-G-C	0.253	0.279	0.326	0.309	0.267	0.254	0.719	0.719	0.252	0.3	0.210	0.229
G-A-C	0.017	0.041	0.014	**0.014**	0.043	0.027	0.219	0.257	7.407E−3	0.039	0.012	**0.016**
G-G-T	6.61E−3	0.019	0.087	0.051	0.019	8.969E−3	0.423	0.306	3.704E−3	0.022	0.034	0.065

[a]The haplotypes are formed by rs3762977, rs2303751, rs121913540, rs121913541, and rs1017.

[b]The haplotypes are formed by rs2303751, rs121913540, and rs121913541; *p*-values are based on 1000 permutations; bold font presents $p < 0.05$.

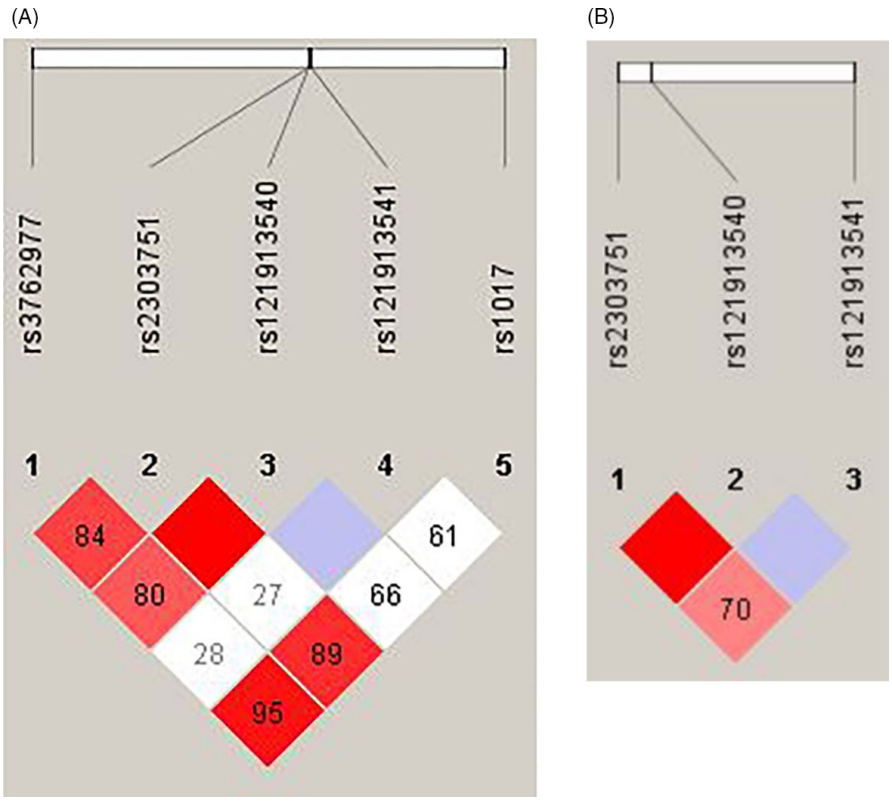

FIGURE 3 Linkage disequilibrium (LD) analysis of ISL1. LD plots show pairwise LD between analyzed SNPs, displayed by standard color schemes (LD = 0, white; LD = 100, Dark red). (A) LD plot for 5 SNPs rs3762977, rs2303751, rs121913540, rs121913541, and rs1017. (B) LD plot for 3 SNPs in the coding region rs2303751, rs121913540, and rs121913541. D' values are represented as percentages with in squares. D' = 1 complete LD, D' > 0.75 strong LD, 0.5 < D' > 0.74 moderate LD, D' < 0.49 weak LD, D' = 0 absence of LD

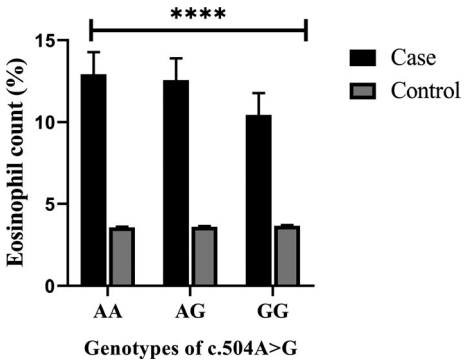

FIGURE 4 Eosinophil percentage in blood was significantly associated with ISL1 c.504A>G polymorphism (****$p < 0.0001$, ANOVA) and was found elevated in OM patients with AA genotype compared to AG and GG genotypes

inner ear). The middle ear epithelium and eosinophilic interaction may elicit further inflammatory responses, would release active oxygen species and cytotoxins like eosinophilic cationic protein which is responsible for tissue damage of middle and inner ear.[35] This study found that OM patients with "GG" genotype for c.504A>G may be less susceptible to the eosinophilic damage due to reduced eosinophilic percentage in circulation when compared to its other genotypes.

Although the study brought out the allelic, genotypic, haplotype, and eosinophilic association with the identified *ISL1* variants, further transcriptional and translational studies on the effect of the identified synonymous variants on the clinical condition of the disease would give out more information. Still larger sample size might have provided a better picture of the study.

Ours is the first report which shows a significant association of *ISL1* variants (rs2303751 and rs121913540) with hearing-related disorders like CSOM in humans. The haplotype and the subsidiary eosinophilic association to particular alleles or genotypes, respectively, further strengthen the protective or risk role of these polymorphisms in the etiopathology of the disease. Further transcriptional and translational studies of these variants in humans may provide the actual mechanisms and pathways involved which may be useful for a better understanding of the etiopathology of otitis media and render newer molecular targets for drug development.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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