Otology

The rs39335 polymorphism of the *RELN* gene is not associated with otosclerosis in a southern Italian population

Il polimorfismo rs39335 del gene RELN non è associato con l'otosclerosi in una popolazione del Sud Italia

S. IOSSA[1 2], V. CORVINO[1], P. GIANNINI[1], R. SALVATO[1], M. CAVALIERE[1], M. PANETTI[3], G. PANETTI[4], B. PIANTEDOSI[4], E. MARCIANO[1], A. FRANZÈ[2 5]

[1] Unit of Audiology, Department of Neurosciences, University "Federico II", Naples, Italy; [2] Institute of Genetics and Biophysics "A. Buzzati Traverso", CNR, Naples, Italy; [3] Department of Otorhinolaryngology, University "Federico II", Naples, Italy; [4] UOC Otorhinolaryngology, PO Ascalesi, Naples, Italy; [5] CEINGE-Biotecnologie Avanzate, Naples, Italy

SUMMARY

Otosclerosis, the single most common cause of hearing impairment in white adults, is characterised by bone dystrophy localized to the otic capsule and isolated endochondral bone sclerosis with alternating phases of bone resorption and formation. Conductive hearing loss develops when otosclerotic foci invade the stapedio-vestibular joint (oval window) and interfere with free motion of the stapes, but affected subjects frequently develop profound sensorineural hearing loss. The aetiology of otosclerosis is unknown. In the last years, several association studies have been performed and have suggested that single nucleotide polymorphisms in some genes may be implicated in development of otosclerosis. The strongest association has been demonstrated for the *reelin* gene, located on chromosome 7q22.1, which encodes an extracellular matrix protein. The involvement of *reelin* in the pathogenesis of otosclerosis is controversial; it was identified in European and North African populations, but was excluded in an Indian population. To analyze the role of *reelin* in otosclerosis, it has been studied in a case-control analysis for the polymorphism rs39335 in a southern Italy population. In this population, the pathogenic link between the rs39335 variant and otosclerosis was excluded.

KEY WORDS: RELN gene • Otosclerosis • Single nucleotide polymorphism • rs39335

RIASSUNTO

L'otosclerosi, la causa più comune di perdita uditiva tra gli adulti bianchi, è caratterizzata da distrofia ossea localizzata alla capsula otica e sclerosi ossea encondrale isolata con alternanza di fasi di riassorbimento e formazione dell'osso. L'ipoacusia trasmissiva si sviluppa quando i focolai otosclerotici invadono l'articolazione stapedio-ovalare (finestra ovale) ed interferiscono con il libero movimento della staffa, ma spesso i soggetti affetti sviluppano un'ipoacusia neurosensoriale profonda. Ad oggi, l'eziologia dell'otosclerosi non è ancora nota. Negli ultimi anni, studi di associazione di diversi polimorfismi sono stati eseguiti e hanno suggerito il coinvolgimento di alcuni di essi nell'insorgenza dell'otosclerosi. L'associazione più forte è stata dimostrata per il gene relina, localizzato sul cromosoma 7q22.1 codificante una proteina della matrice extracellulare. Il coinvolgimento di relina nella patogenesi dell'otosclerosi è controverso in quanto è stato identificato in alcune popolazioni Europee e del Nord Africa, ma è stato escluso nella popolazione Indiana. Per analizzare l'importanza di relina nell'otosclerosi è stato realizzato uno studio di associazione caso-controllo per il polimorfismo rs39335 in una popolazione del Sud Italia. In questa popolazione l'associazione del polimorfismo rs9335 nello sviluppo dell'otosclerosi è stata esclusa.

PAROLE CHIAVE: *Gene RELN • Otosclerosi • Polimorfismo a singolo nucleotide • rs39335*

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Introduction

Otosclerosis (OTSC) is the single most common cause of hearing impairment among white adults with a prevalence of 0.3-0.4% in the European population [1]. Otosclerosis is characterized by bone dystrophy localised to the otic capsule and isolated endochondral bone sclerosis with alternating phases of bone resorption and formation: mature lamellar bone is removed by osteoclasts and replaced by osteoblasts with a thicker, more vascular bone.

Conductive hearing loss develops when otosclerotic foci invade the stapedio-vestibular joint (oval window) and interfere with free motion of the stapes, but about 10% of

affected subjects develop profound neurosensorial hearing loss across all frequencies [2].

Mean age of onset is between the third decade, and 90% of affected individuals are under 50 years of age at the time of diagnosis. The aetiology of otosclerosis is unknown, although several theories have been postulated. For the familiar forms, which present a dominant form of transmission with reduced penetrance eight loci have been identified: OTSC1 (15q25-26) (OMIM 166800), OTSC2 (7q34-36) (OMIM 605727), OTSC3 (6p21.2-22.3) (OMIM 608244), OTSC4 (16q21-23.2) (OMIM 611571), OTSC5 (3q22-24) (OMIM 608787), OTSC7 (6q13-16.1) (OMIM 611572), OTSC8 (9p13.1-9q21.11) (OMIM 612096) and OTSC10 (1q41-44), but no causative gene has been yet identified [3-10]. Moreover, in the last years several association studies have been performed and have suggested the implication of single nucleotide polymorphisms (SNPs) present in several genes in otosclerosis aetiology [11-15]. For some genes the results are controversial.

In particular, an interesting and controversial case is represented by the *reelin (RELN)* gene which was associated in some populations, but not in others [14 16-18]. The *RELN* gene, located on chromosome 7q22.1 and encoding the extracellular matrix protein reelin, is expressed by neural tissues [19 20]. The results regarding *reelin* expression in the inner ear and in human stapes footplates are also controversial [14 21], making the aetiologic role of *RELN* in the pathogenesis of otosclerosis unclear. In previous studies, among the identified single nucleotide polymorphisms (SNPs) in *RELN* gene, the rs39335 variant was one of the most promising [16 17]. In order to add information about a possible role of *RELN* in the aetiology of otosclerosis, we performed a case-control association study in a southern Italian population for the SNP rs39335.

Materials and methods

Patient selection

A total of 92 otosclerotic subjects were recruited by clinical centres present in Naples (Audiology and Othorhinolaryngology Units of University of Naples "Federico II"; Othorhinolaryngology Unit of "C. Ascalesi" Hospital, Naples, Italy). Subjects (65 females and 27 males) were all unrelated: age range 30-50 years. For 51 patients, clinical diagnosis was based on surgical findings during stapes surgery, while for the 41 remaining patients was based on audiological data [22 23]. The control group was composed of 92 healthy individuals. Affected and control subjects are all originated from the Campania region. Written informed consent was obtained from all participants for DNA analysis according to the principles of the Helsinki Declaration.

SNP analysis and genotyping

Genomic DNA was extracted by conventional salt precipitation protocols from peripheral blood samples obtained in EDTA-containing tubes. For genotyping the rs39335 SNP in *RELN* the following primers were used (RELN1F 5'-GTCAATGATGAGGATGTTAATGTATA and RELN1R 5'-GAGAGAGACTAGCCAGAGGATC-3'): the RELN1F primer introduces a recognition site for the restriction enzyme Bstz17I. The primer pairs was designed using the PIRA PCR programme at http://cedar.genetics.soton.ac.uk/public_html/primer2.html. To amplify the region of interest in *RELN*, a polymerase chain reaction (PCR) was performed using 50 ng of purified genomic DNA in a PCR mix containing 10X Buffer II, 25 mM $MgCl_2$, 5 U/µl Ampli Taq Gold; (Applied Biosystems) in the presence of 2.5 mM deoxynucleotide triphospate (dNTP) and 25 mM primers. For the *RELN* gene PCR was carried out with an initial denaturation cycle of 95°C for 10 min, 38 cycles with denaturation at 95°C for 1 min, annealing at 54°C for 1 min, elongation at 72°C for 1 min, and finally elongation at 72°C for 10 min. PCR products were digested overnight at 37°C according to the manufacturer's instructions.

Statistical analysis

The association of the rs39335 SNP with otosclerosis was analyzed by an exact chi-square test, comparing SNP frequencies using the software FINETTI (http://ihg2.helmholtz-muenchen.de.). Hardy-Weinberg equilibrium (HWE) of tested groups and Amirtage's trend test (ATT) as well as allele and genotype frequencies and odds ratios were also calculated using the FINETTI software.

Significant differences were considered to be when $p < 0.05$. Genotype and allele frequencies were compared between the two groups (otosclerotic subjects and healthy controls).

Results

Case-control analysis for the SNP rs39335

To add information about a potential pathogenic link between a rs39335 SNP and otosclerosis, a case-control study in 92 patients and 92 controls was performed using a restriction enzyme assay for the presence of the polymorphism rs39335, which was one of the more strongly associated SNPs in previous studies [16 17], with the G allele frequency higher in otosclerotic subjects than in controls. Table I shows the distribution of G allele frequencies in OTSC patients and healthy controls. In the examined population, it was less frequent in otosclerotic subjects than in controls. Table II shows the frequencies of the different genotypes (AA, AG, GG) in otosclerotic and control subjects. The AG and GG phenotypes were more frequent in control subjects, while the AA genotype was more frequent in otosclerotic subjects.

The frequency of the G allele was 16% (29/184 alleles) in otosclerotic individuals compared to controls in which the frequency was 23% (42/184 alleles) (Table I). The difference in genotype distribution for the rs39335 SNP did

Table I. Frequencies of the G allele between otosclerotic patients and control subjects.

SNP	Position	Sequence change	Frequency in control subjects	Frequency in otosclerotic patients
rs39335	28637145	A > G	42/184 (23%)	29/184 (16%)

Table II Genotype distribution of the *RELN* rs39335 SNP in otosclerotic patients and control subjects.

	Genotype frequency		
	AA	AG	GG
Control	54 (59%)	34 (37%)	4 (4%)
OTSC	65 (71%)	25 (27%)	2 (2%)

not reach statistical significance (p = 0.0813; OR = 0.626; 95%CI: 0.37-1.07).

Discussion

Otosclerosis is a complex disease; although several studies have been carried out, the aetiopathogenesis of otosclerosis remains poorly understood. In the last years, several association studies suggesting that nucleotide variations in some genes may predispose to otosclerosis have been carried out [11-15]. One of the genes analyzed was *RELN* for which a strong association in French, Belgian-Dutch German, Swiss, Romanian and northern Italian populations was demonstrated [14 16 17]. This association was, however, not confirmed in an Indian population [18]. Moreover, other data supporting the exclusion of the *RELN* gene as causative for otosclerosis are reported in a recent paper [21] where it was shown that this gene does not show active expression in adult stapes footplates.

Confirmatory studies in genotype-phenotype association are important for establishing the credibility of results. In fact, frequently, initial results cannot be confirmed. In this case-control study, both allele (Table I) and genotype (Table II) distributions of one of the most associated variants in previous works [16 17], the SNP rs39335, show no statistically significant association, suggesting that the rs39335 SNP is not a risk factor for otosclerosis.

Conclusions

The results obtained in this study show that, in the Campania population, there is no association between the rs39335 SNP and otosclerosis. Other variants, however, may still play a role in the disease. Further studies analyzing other variants and additional populations of different ethnic origin would be useful to verify the exact role of *RELN* in development of otosclerosis.

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Address for correspondence: Annamaria Franzè, Institute of Genetics and Biophysics "A. Buzzati Traverso", CNR, via Pietro Castellino 111, 80131 Napoli, Italy. E-mail: franze@igb.cnr.it