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ORIGINAL ARTICLE

Exclusion of *TNFRSF11B* as Candidate Gene for Otosclerosis in Campania Population

Sandra Iossa · Giovanna Morello · Teresa Esposito · Virginia Corvino · Pasquale Giannini · Raffaella Salvato · Michele Cavaliere · Maria Panetti · Giuseppe Panetti · Bruno Piantedosi · Fernando Gianfrancesco · Elio Marciano · Annamaria Franzè

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Abstract The etiology of otosclerosis is unknown. The etiopathogenesis of otosclerosis seems similar to that occurring in Paget's disease of bone, for which mutations or polymorphisms in several genes have been identified. Among these, *TNFRSF11B* gene encoding the osteoprotegerin is produced at high levels in the normal inner ear and at low level in active otosclerotic stapes footplates. The aim of this work was to verify the presence of a correlation between the rs2073618 (N3K) polymorphism in the *TNFRSF11B* gene and otosclerosis. Mutational screening in the *TNFRSF11B* gene was performed by direct sequencing. SNPs analysis was performed by PCR and by specific restriction enzyme assay with HpaI. The significance of the association was analyzed by statistical specific software. No causative mutation has been identified but the data suggested a strong correlation between the rs2073618 (N3K) polymorphism and otosclerosis. This correlation, however, has been excluded in a case–control study. This study excluded the association between the N3K polymorphism and otosclerosis in Campania region population.

Keywords Osteoprotegerin · Case–control study · SNP · Rs2073618 · Campania region

S. Iossa · V. Corvino · P. Giannini · R. Salvato · E. Marciano
Unit of Audiology, Department of Neurosciences, Reproductive and Odontostomatologic Sciences, University of Naples "Federico II", Naples, Italy

S. Iossa · A. Franzè (✉)
CEINGE-Biotecnologie Avanzate, Via G. Salvatore 486, 80145 Naples, Italy
e-mail: franze@igb.cnr.it

G. Morello · T. Esposito · F. Gianfrancesco · A. Franzè
Institute of Genetics and Biophysics "A. Buzzati Traverso", C.N.R., Naples, Italy

G. Morello
Institute of Cybernetics "Eduardo Caianello", Naples, Italy

M. Cavaliere · M. Panetti
Department of Otorhinolaryngology, University of Naples "Federico II", Naples, Italy

G. Panetti · B. Piantedosi
U.O.C. Otorhinolaryngology, PO Ascalesi, Naples, Italy

Introduction

Otosclerosis is a disorder of the labyrinth bone, that only affects humans, causing conductive, sensorineural or mixed hearing loss [1]. The age-of-onset ranges from 15 to 40 years, with an average onset occurring in the third decade [2] and with a prevalence of 0.3–0.4 % in the European population [3]. Several studies aimed at determining the factors involved in the etiology of the disease have been performed, and different hypotheses are still under evaluation [4–6]. Linkage analyses performed on large families with dominant transmission and incomplete penetrance, have led to the identification of eight monogenic loci (http://hereditaryhearingloss.org/) [7–14]. However, to date no causative gene has been identified. Moreover, several association studies have suggested the implication of SNPs located in several genes [15–20]. The loci identified by linkage analysis and the genes putatively implicated through association studies do not overlap. The mechanism and the etiopathogenesis of otosclerosis show some similarities to those occurring in Paget's disease of bone (PDB) [21–24]. In the etiology of PDB genetic factors play an important role, and mutations or polymorphisms in several disease-associated genes have been identified [25–30], including the tumor necrosis factor receptor super-family member 11b (*TNFRSF11B*) encoding the

osteoprotegerin (OPG). This gene plays a key role in the *NF–kB* signaling pathway and it is likely that mutations in this gene predispose to PDB by disrupting physiological signaling, leading to osteoclast activation [31]. Knockout mice for *TNFRSF11B* gene have an altered auditory function [32]. The OPG protein, which inhibits the activation and function of osteoclasts, is produced at high levels in the inner ear of normal mice and it is secreted into the perilymph. Human otic capsule is characterized physiologically by high OPG expression, since bone turnover is almost completely absent in the bone adjacent to the perilymphatic space [33]. Recently, a significantly low level of OPG in active human otosclerotic stapes footplates has also been demonstrated [24]. Mutations in *TNFRSF11B* gene have been identified as causative of JPG (juvenile Paget's disease), and there is also evidence that polymorphisms in this gene contribute to an increased susceptibility to the classic form of PDB [34]. Previous studies have excluded association with some of the already known loci for affected subjects originating from the Campania region [35]. In this study, given the shared clinical manifestations between otosclerosis and Paget's disease, a mutational screening in the *TNFRSF11B* gene in 12 patients affected by otosclerosis was performed and a successive case control analysis carried out on ninety-eight affected subjects.

Methods

Selection of Subjects and Clinical Evaluation

The affected subjects (98) enrolled in this study were recruited by clinical centers present in Naples (Audiology and Othorhinolaryngology Units of University of Naples "Federico II"; Othorhinolaryngology Unit of "C. Ascalesi" Hospital, Naples, Italy). The subjects (69 females and 29 males) were all unrelated: age range 30–50 years. Pedigree information was obtained to determine the type of transmission of hearing loss. In 51 patients, the clinical diagnosis was based on surgical findings during stapes surgery while diagnosis was based on audiological data with a diagnostic protocol, performed according to international standards, for the remaining 47 subjects [35]. The control group was composed of 100 healthy subjects originating from Campania. Genomic DNA was extracted by means of conventional salt precipitation protocol from peripheral blood samples obtained in EDTA-containing tubes. Written informed consent for DNA analysis was obtained from all participants according to the principles of the Helsinki Declaration.

Molecular Screening

Mutational analysis, in the five exons of *TNFRSF11B* gene, was performed by direct sequencing. The regions of interest were amplified by Polymerase Chain Reaction (PCR) using 50 ng of the purified genomic DNA in a PCR mix containing Buffer II 10×, $MgCl_2$ Solution 25 mM, Ampli Taq Gold (Applied Biosystems) 5U/mL in the presence of 2.5 mM deoxynucleotide triphospate (dNTP) and 25 mM sense and antisense primers. PCR was developed with an initial denaturation cycle of 95 °C for 10 min, 38 cycles with denaturation at 95 °C for 1 min, annealing at the temperature set for each primer pair for 1 min and elongation at 72 °C for 1 min. Finally elongation was set at 72 °C for 10 min. Coding exons of *TNFRSF11B* genes were amplified using two pairs of primers for each exon, located in the flanking introns (Table 1). Primers were designed by "Oligo 4" software. PCR products were run on 2 % agarose gel with ethidium bromure. Each purified PCR product was sequenced and the sequences were analyzed by alignment with "Autoassembler 2.1" software.

SNP Analysis and Genotyping

Modified primers (Table 2) were used to genotype SNP rs2073618 in *TNFRSF11B* gene: the reverse primer introduces a recognition site for the restriction enzyme HpaI. PCR products were digested for 3 h at 37 °C according to the manufacturer's instructions.

Statistical Analysis

The association of rs2073618 SNP, located in *TNFRSF11B* gene, with otosclerosis was analyzed by exact Chi square test, comparing SNP frequencies using software FINETTI (http://ihg2.helmholtz-muenchen.de.). Hardy–Weinberg equilibrium of tested groups, Amirtage's trend test, allele and genotype frequencies and O.R. were calculated using the same software. Genotype and allele frequencies were compared between the groups (otosclerotic patients and healthy controls).

Results

Mutational Analysis

A mutational analysis on 12 individuals affected by otosclerosis (6 females and 6 males) was performed. The family pedigree for all the examined individuals revealed a dominant transmission of the pathology. All exons of *TNFRSF11B* gene, including about 100 bp of flanking intronic regions, were analyzed using the sequences of *TNFRSF11B* gene (chr8: 19935796–119964383; NM_002546) from the NCBI (http://genome.ucsc.edu/) as reference sequences. No pathological mutation has been identified in the examined gene in any of the analyzed

Table 1 Primers used for amplification of TNFRSF11B coding region

	Forward 5′→3′	Reverse 5′→3′
Exon 1	TGCCGGGACGCTATATATAAC	TTCTCCCCGCCGGTCCGCT
Exon 2	ATCTGCATTCCTGGTCTTTGA	TCCTCTGAGCAATGGTCCTT
Exon 3	AAGGGGATGATGGTGGAAGT	GCTGGTTAAGATTCAAGAAAGG
Exon 4	CACTAAGACCAGCCAACAGAA	ACAAGATCCACACAACTAAACA
Exon 5	TTTTGCCTCACGCTTGTTTTAT	TCCTTTCTCCACATCATAGTTT

Table 2 Primers used for genotyping assay of rs2073618 (N3 K)

	Forward (5′→3′)	Reverse (5′→3′)
Exon 1	CCCTGAAAGCGTTAATCCTGGAGC	GGGACTTACCACGAGCGCGCAGCACGTTAA

patients. However, two synonymous and one non synonymous variations were found (L56L, S77S, N3 K). Interestingly, the rs2073618:C>G polymorphism, responsible for the amino acid change N3K, in the exon 1 of *TNFRSF11B* gene, was very frequently found among screened otosclerotic patients (10/12; 83 %) (Table 3).

Case–control analysis and statistical analysis

To determine whether the SNP rs2073618 has a significant association with the otosclerosis in the Campania population a case–control study was performed, by specific restriction enzyme assay with HpaI, in a total of 98 patients and a control group: 100 unaffected subjects originating from the Campania region. Among the 98 otosclerotic subjects, 56 were sporadic cases and the family pedigree of 42 subjects revealed dominant transmission of hearing loss. In otosclerotic subjects the frequency of G allele is 57 % (111/196) while in the controls it is 51 % (102/200) (Table 4). The *p* value obtained with the Amirtage's trend test results not significant with the control group (Table 4).

Table 3 Variations identified in the *TNFRSF11B* gene in 12 otosclerotic subjects

Nucleotide position	Aminoacid position	N° subjects
c.1181 C>G	N3K	5
c.1403 T>C	S77S	1
c.1181 C>G+c.1403 T>C	N3K+S77S	3
c.1181 C>G+c.1940 A>G	N3K+L56L	2

Discussion

Otosclerosis can be considered a multifactorial disease. From a genetic point of view, although some loci have been mapped, no causative gene has been identified. Association studies show that nucleotide variation in some genes may predispose to otosclerosis [15–19]. Nonetheless the etiopathogenesis of otosclerosis remains unclear [4–6]. However, bone remodeling, dramatically altered in otosclerotic patients, is quite similar to what occurs in Paget's disease of bone (PDB). Interestingly OPG protein, involved in PDB, is a potent inhibitor of osteoclastogenesis. It is expressed at high levels within the inner normal ear and is secreted into the perilymph and the surrounding bone and may serve to inhibit active bone remodeling within the otic capsule, especially immediately adjacent to the cochlea [33]. OPG involvement in bone remodeling in otosclerosis is also suggested by the identification of low level of OPG expression in active human otosclerotic stapes [24].

Mutations in this gene have been identified in patients with juvenile Paget's disease, which develop over time otosclerosis too [23]. Prompted by these observations, a molecular screening of *TNFRSF11B* gene was carried out. The sequencing data did not reveal the presence of any causative mutations excluding *TNFRSF11B* gene as a common cause of the disease for all 12 otosclerotic subjects.

Table 4 Statistical relationship between OTSC subjects and healthy Campania region controls for rs2073618

	n	Allele frequency		Amirtage's trend test (*p*)	Odds ratio (OR) (95 % CI)	Genotype frequency		
		C (%)	G (%)			CC (%)	CG (%)	GG (%)
Controls	100	98 (49)	102 (51)			28 (28)	42 (42)	30 (30)
OTSC patients	98	85 (43)	111 (57)	0.27384	1.261 (0.845–1.864)	17 (17)	51 (52)	30 (31)

However three nucleotide variations (SNPs) were identified in several subjects (Table 3). In particular the SNP rs2073618 (N3K) resulted very frequent among the analyzed otosclerotic subjects. By a case–control study, a statistically significant association was not found between this SNP and otosclerosis ($p = 0.27384$) (Table 4) excluding a role for *TNFRSF11B* gene in otosclerosis in the Campania region population and also confirming data reported in previous studies in other populations (Belgian, Dutch and French otosclerotic subjects) that reported negative results on screening for SNPs in TFNRSF11B gene [18].

Since several other genes are involved in different bone-related pathologies, such as osteoporosis, otosclerosis and Paget disease of bone, other genes involved in *NF–kB* signaling pathway need to be investigated for mutations in otosclerosis in the future, in order to ascertain their possible involvement in the etiology of these diseases.

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Conflict of interest The authors declare that there is no conflict of interest.

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