Human Molecular Genetics, 2007, Vol. 16, No. 17 **2021–2030**
doi:10.1093/hmg/ddm150
Advance Access published on June 22, 2007

The coding polymorphism T263I in TGF-β1 is associated with otosclerosis in two independent populations

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Received May 8, 2007; Revised and Accepted June 8, 2007

Otosclerosis is a progressive hearing loss characterized by an abnormal bone homeostasis of the otic capsule that leads to stapes fixation. Although its etiology remains unknown, otosclerosis can be considered a complex disease. Transforming growth factor-beta 1 (TGF-β1) was chosen for a case–control association study, because of several non-genetic indications of involvement in otosclerosis. Single nucleotide polymorphism (SNP) analysis in a large Belgian–Dutch sample set gave significant results ($P = 0.0044$) for an amino acid changing SNP, T263I. Analysis of an independent French population replicated this association with SNP T263I ($P = 0.00019$). The results remained significant after multiple testing correction in both populations. Haplotype analysis and the results of an independent effect test using the weighted haplotype (WHAP) computer program in both populations were both compatible with SNP T263I being the only causal variant. The variant I263 is under-represented in otosclerosis patients and hence protective against the disease. Combining the data of both case–control groups for SNP T263I with a Mantel–Haenszel estimate of common odds ratios gave a very significant result ($P = 9.2 \times 10^{-6}$). Functional analysis of SNP T263I with a luciferase reporter assay showed that the protective variant I263 of TGF-β1 is more active than the WT variant T263 ($P = 1.6 \times 10^{-6}$). On the basis of very low P-values, replication in an independent population and a functional effect of the protective variant, we conclude that TGF-β1 influences the susceptibility for otosclerosis, and that the I263 variant is protective against the disease.

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INTRODUCTION

Otosclerosis is a common bone dysplasia that is unique to the endochondral layer of the otic capsule. Otosclerosis is a disease particularly frequent among the white population, whereas it is very rare among Blacks, Asians and Native Americans (1). Schuknecht and Barber (2) classified otosclerosis as clinical and histological. Histological otosclerosis has an estimated prevalence of 2.5%, and denotes a disease process in which clinical symptoms are absent, and can only be discovered by CT-scanning or sectioning of the temporal bone at autopsy. Clinical otosclerosis, in contrast, is caused by otosclerotic lesions that impinge on stapes mobility, thereby causing a progressive conductive hearing loss. The prevalence of clinical otosclerosis is estimated at 0.3–0.4% in the Caucasian population and is twice as frequent in females as in males (3).

The otic capsule may be 'singled out' as a disease site in otosclerosis because it is unique among bone. The otic capsule undergoes very little remodeling of bone (only 0.13% per year) after development (4,5). Recently, researchers found that this specific inhibition at the otic capsule is due to intrinsic factors produced by the cochlea (6–8). However, in otosclerosis there is an abnormal balance between bone resorption and bone deposition, and bone remodeling within the otic capsule occurs more rapidly (4,5). The biological mechanisms that control this special bone metabolism in the otic capsule remain largely unknown at present.

Many theories about the etiology of otosclerosis have been postulated. However, the true nature about the origin of otosclerosis still remains unclear. From a genetic viewpoint, otosclerosis is a multi-factorial disease with rare autosomal dominant forms. About 50% of patients report no other affected family members, and in instances in which the family history is positive, pedigrees are rarely large enough for genetic linkage analysis. Factors like reduced penetrance and phenocopies often complicate genetic analysis of otosclerosis. To date six autosomal dominant loci have been published (9–14).

In contrast to the scarcity of large monogenic otosclerosis families, sporadic cases of otosclerosis are very common. A case–control association study is a very suitable method to search for genes involved in multi-factorial diseases. The only association studies performed thus far for otosclerosis have analyzed the *COL1A1*, *COL1A2* and *COL2A1* genes and have generated conflicting results (15–18). Possible environmental factors that can contribute to the etiology of otosclerosis include viruses like measles, and hormonal factors like estrogens, but corresponding studies have generated inconsistent results (19–23). Some researchers suggest that the trigger of the disease process is an autoimmune reaction of the endochondral otic capsule to the 'globuli interossei' (19,24,25).

Transforming growth factor-β1 (TGF-β1) is the prototype of the TGF-β superfamily that includes TGF-βs, the bone morphogenetic proteins (BMPs) and activins. TGF-β1 is a multi-functional growth factor that regulates a broad range of biological processes in many cell types, including cell proliferation, differentiation, migration and production of extracellular matrix (26). It is synthesized as a precursor structure (pre-pro-TGF-β1) consisting of three distinct parts: the signal peptide (29 amino acids), the latency-associated peptide (LAP; 249 amino acids) and the mature peptide (112 amino acids).

Pre-pro-TGF-β1 monomer is extensively processed before its secretion. Initially, the signal peptide is cleaved-off and pro-TGF-β1 dimers are formed (27). The LAP and the mature peptide are non-covalently bound to each other, conferring latency to the mature peptide. This latent complex is stored in the extracellular matrix and is an important way of controlling biological activity since expression is ubiquitous (28,29). For full biological activity, TGF-β1 needs to be activated. Once activated, TGF-β1 can bind to its receptors and regulate gene transcription through the Smad pathway (30,31).

Bone is a dynamic tissue that is continuously remodeled by the balanced process of bone resorption and formation. TGF-β1 is stored in a latent state in the bone matrix. It has been hypothesized that resorbing osteoclasts can activate the latent form of TGF-β1 in the acidic microenvironment of their ruffled border (31). Active TGF-β1 will in turn inhibit further bone resorption by impairing osteoclast differentiation and activation, and promote bone formation by stimulating osteoblast chemotaxis, proliferation and differentiation (32). Although the details are still not clarified, it can be concluded that TGF-β1 plays a pivotal role in bone-remodeling, coupling bone resorption to new bone formation.

TGF-β1 also plays a role in embryonic development of the ear. During embryonic formation of the otic capsule, TGF-β1 plays a role as a regulatory molecule that is very important in the epithelial–mesenchymal interactions and the epithelial induction of the otic capsule chondrogenesis. In an early stage of inner ear development, the otic epithelium produces TGF-β1 that stimulates the chondrogenesis of the mesenchyme to promote otic capsule growth. In a later stage, TGF-β1 selectively inhibits this process to permit perilymphatic space formation and capsular modeling (33–35). TGF-β1 expression was also observed in specimens of adult human otosclerotic and normal bone consisting of the stapedial footplate, suggesting a role for this signal molecule in the bone turnover and remodeling of the otic capsule (33–35). Consistent with this possibility, another study reported a difference in phenotypic expression of glycosaminoglycans, fibronectin and collagen in the extracellular matrix between adult normal and otosclerotic bone cells, and these differences can be modified by TGF-β1 treatment (36,37). Many studies can be found in the literature that suggest that TGF-β1 is implicated in the pathogenesis of otosclerosis (33–40), but proof is still lacking.

In this study, we have completed a case–control association study between the otosclerosis phenotype and TGF-β1 variants in two independent populations. TGF-β1 was chosen as a primary candidate gene because of its importance in the bone metabolism of the otic capsule, and because of the suggestions in the literature of its involvement in otosclerosis. For one TGF-β1 variant (T263I) we present a significant difference in genotype frequencies between otosclerosis patients and controls as well as functional evidence that TGF-β1 confers susceptibility for otosclerosis.

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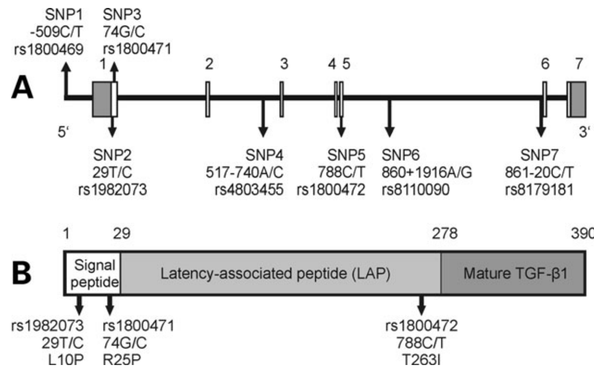

Figure 1. Schematic representation of the *TGF-β1* gene (**A**) and the pre-pro-TGF-β1 (**B**). (A) The boxes represent the seven numbered exons and the 5′- and 3′-untranslated region (UTR) are marked in gray. Analyzed SNPs are marked with an arrow; the first line gives the SNP number, the second line the SNP position and the base substitution starting from the start codon, and the third line is the SNP name (rs-number) from NCBI. (B) The numbers above indicate the amino acid position of the cleavage sites of the pre-pro-TGF-β1. The amino acid changing SNPs are shown: the first line is the rs-number, the second line indicates the base substitution and the third line the amino acid substitution both starting from the start codon.

RESULTS

Genetic analyses

Association study in the Belgian–Dutch case–control group. A total of seven SNPs in TGF-β1 were analyzed in the Belgian–Dutch (B–D) case–control group, including the optimal set of tagSNPs retrieved from the International HapMap Project database (www.hapmap.org; SNPs 1, 4 and 6), supplemented with possible functional SNPs based upon information in the literature (SNPs 2, 3, 5 and 7; Fig. 1; Table 1). The tagSNPs covered more than 90% of the alleles in the region with r^2 value of 0.8 or greater (41). The SNPs were genotyped in 632 otosclerosis patients from Belgium or the Netherlands and 632 control individuals at these SNP loci (Table 2). All SNPs were in Hardy–Weinberg equilibrium (HWE). To test the association between the candidate SNPs and otosclerosis, we performed logistic regression, whereby the genotype was coded linearly, testing for significance using the likelihood ratio test (LRT). Since otosclerosis has a higher prevalence in women compared with men (42), we also tested the possibility that some SNPs have a different effect for males and females.

We found two SNPs significantly associated with otosclerosis. The first SNP (SNP 5) was an amino acid changing SNP, T263I, located in exon 5 in the LAP of TGF-β1. This SNP has a *P*-value of 0.0044 and an odds ratio (OR) of 0.423 with a 95% confidence interval (CI) of 0.228–0.784. The rare allele of the associated SNP T263I (allele leading to I at position 263) was more frequent in the control population (5.9% in controls and 2.4% in patients). Homozygotes for the I allele were never seen. The second associated SNP in the B–D population (SNP 7) is located just 20 bases before exon 6 and has a *P*-value of 0.019 and an OR of 0.804 (0.670–0.965). Logistic regression analysis showed no evidence for effect modification of sex on the SNP effect in any of the SNPs tested (data not shown).

With the weighted haplotype (WHAP) program (43), haplotypes were inferred (SNP 2, 3, 5, 7) and tested for association. The overall haplotype analysis gave a *P*-value of 0.0296. Testing the haplotype CGTC against all other haplotypes, revealed a *P*-value of 0.0085 (Table 3). This haplotype CGTC is also the only haplotype that contains the associated I263 variant of SNP 5.

Although correcting for multiple testing is still a controversial issue in association studies, we performed both a Bonferroni correction and a permutation test that is implemented in the Haploview program to assess statistical significance of the results. Only SNP T263I survived both multiple testing corrections (Table 2) The Bonferroni corrected cut-off *P*-value is 0.007 (0.05/7 SNPs). The haplotype CGTC was still significant after the permutation test (Table 3). SNP 7 was no longer significantly associated with otosclerosis.

With the WHAP program we performed the independent effect test, which is a conditional test that can determine which variant (SNP) is most likely to be causal, as opposed to showing only indirect association due to linkage disequilibrium (LD) with the causal variant. This test investigates if the SNP is associated with the disease conditional on the other SNPs. The test showed that the significance of SNP T263I remains when controlling for the genotype of the surrounding SNPs, whereas the significance of SNP 7 dropped. This is compatible with T263I being the only causal variant (Table 4).

Confirmation of the association in a French case–control group. To confirm this association, we sought to replicate our association in an independent population. Therefore, we collected an independent sample of 457 French otosclerosis patients and 497 French controls and only analyzed SNPs 2, 3, 5 and 7. Significant results (Table 5) were found in this population for SNP L10P with a *P*-value of 0.012 and an OR of 1.260 (1.051–1.510), and SNP T263I with a *P*-value of 0.00019 and an OR of 0.334 (0.180–0.620). The overall *P*-value for the haplotype analysis was 0.00144 (Table 6). We found a significant result for the same haplotype as in the B–D case–control group (CGTC) with a *P*-value of 0.0003.

Moreover, the association with SNP T263I, but not SNP L10P, survived both corrections for multiple testing (Bonferroni cut-off *P*-value 0.013; Table 5). The haplotype CGTC also stays significant after permutation (Table 6). In addition, the independent effect test was compatible, with SNP T263I being the only causal variant (Table 4). This analysis therefore confirmed the association we found in the B–D population.

In aggregate, these data are consistent across two independent populations, with the same allele in the same SNP (T263I) and the same haplotype (CGTC) showing an association with otosclerosis in both populations. This replication in our independent population strongly supports the true nature of the association. These analyses suggest that this association may be present in other populations of European descent.

Combined analysis of Belgian–Dutch and French case–control group. For the associated SNP (T263I), results from both B–D and French case–control samples were combined

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Table 1. SNP selection and genotyping

SNP number	rs number	Position	Base substitution	Amino acid substitution	SNP selection	Population	
						French	B–D
1	1800469	5′-UTR	−509C → T	Promoter	tagSNP/literature		X
2	1982073	Exon 1	29T → C	L10P	literature	X	X
3	1800471	Exon 1	74G → C	R25P	literature	X	X
4	4803455	Intron 2	517 − 740A → C		tagSNP		X
5	1800472	Exon 5	788C → T	T263I	tagSNP/literature	X	X
6	8110090	Intron 5	860 + 1916A → G		tagSNP		X
7	8179181	Intron 5	861 − 20C → T		tagSNP/literature	X	X

B–D, Belgian–Dutch population.

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Table 2. Case–control association analysis of *TGF-β1* SNPs with otosclerosis in the B–D population

SNP number	Amino acid substitution	Allele frequency (no.)			Odds ratio	Nominal *P*-value	Permutated *P*-value[a]
		Allele	Case	Control			
1	Promoter	C	0.69 (854)	0.68 (827)	0.944 (0.793–1.123)	0.517	0.987
		T	0.31 (376)	0.32 (385)			
2	L10P	T	0.62 (762)	0.61 (731)	1.061 (0.897–1.256)	0.488	0.981
		C	0.38 (454)	0.39 (461)			
3	R25P	G	0.934 (1139)	0.934 (1117)	0.994 (0.720–1.374)	0.973	1.0
		C	0.066 (81)	0.066 (79)			
4		A	0.48 (587)	0.47 (566)	0.958 (0.815–1.127)	0.607	0.995
		C	0.52 (639)	0.53 (642)			
5	T263I	C	0.989 (1205)	0.972 (1174)	0.423 (0.228–0.784)	**0.0044**	**0.030**
		T	0.011 (15)	0.028 (34)			
6		A	0.955 (1173)	0.958 (1155)	1.058 (0.726–1.541)	0.770	0.999
		G	0.045 (55)	0.042 (51)			
7		C	0.76 (933)	0.72 (872)	0.804 (0.670–0.965)	**0.019**	0.110
		T	0.24 (293)	0.28 (340)			

[a]Permutated *P*-value's calculated with the program Haploview, correcting for multiple testing. Significant *P*-values are marked in bold. 95% confidence interval (CI) is given within brackets.

into a Mantel–Haenszel estimate of common allelic OR, and a Cochran–Mantel–Haenszel test for conditional independence (44,45). This revealed a very robust and consistent association with a highly significant *P*-value of 9.2×10^{-6} and an OR of 0.377 (0.243–0.583).

Assessment of population stratification. To assess population stratification that could lead to spurious associations, the *LCT* (lactase gene) SNP-13910C → T, which has a wide variation in allele frequency among closely related populations (46) was analyzed (Supplementary Material, Table S1). According to Campbell *et al*. (47), genotyping this marker can be very informative in detecting subtle, but important levels of population stratification in apparently homogeneous study samples. We found no differences for this SNP in the total B–D population or between sub-populations, separated by origin (Antwerp, Ghent and Nijmegen). Analysis of the LCT SNP in our French sample set also did not show a significant result. Therefore, it is unlikely that the results we obtained are an artifact caused by population stratification.

Functional analyses

The very low *P*-values for association and the replication in an independent population, strongly suggest that TGF-β1 plays a role in the otosclerosis susceptibility in the Western-European population. As the most significant and replicated SNP, T263I changes an amino acid in the LAP of TGF-β1, and the independent effect test suggests that this SNP T263I may be causal, we hypothesized that T263I has a direct causative effect. Our strategy to confirm the causality of the associated SNP T263I was to investigate this variant Ile263 functionally. Using a luciferase reporter assay we sought to compare the activity of the two variants, respectively, Thr and Ile at position 263.

Reporter assay. A pcDNA3 vector containing the TGF-β1 gene product with either the Thr263 variant or the Ile263 variant was co-transfected in HEK293T cells with a pRL-Tk calibration vector and a TGF-β-responsive reporter vector. The TGF-β1 responsive reporter vector contains a construct that consists of 12 CAGA-elements, a minimal promoter and the *Firefly* luciferase gene (48). Only active TGF-β1, and not latent TGF-β1 that is linked to the LAP, will lead to the transcription of *Firefly* luciferase by way of auto- or paracrine activation of the Smad signaling pathway (Fig. 2). Firefly and Renilla luciferase activity were measured in the lysates of the transfected cells (Thr263 and Ile263 variant). Two-way Anova analysis, controlling for differences between the different transfection experiments, was carried out. Despite differences,

Table 3. Haplotype analysis of the possible functional TGF-β1 SNPs with otosclerosis in the B–D population

Haplotypes		Case frequency	Control frequency	Nominal *P*-value	Permutated *P*-value[a]
In base pairs	In amino acid				
TGCC	L10 R25 T263 861-20C	0.447	0.406	*0.051*	0.149
CGCC	P10 R25 T263 861-20C	0.237	0.228	0.629	0.986
TGCT	L10 R25 T263 861-20T	0.180	0.206	*0.089*	0.369
CGCT	P10 R25 T263 861-20T	0.057	0.068	0.156	0.7412
CCCC	P10 P25 T263 861-20C	0.065	0.059	0.490	0.976
CGTC	P10 R25 I263 861-20C	0.012	0.027	**0.0085**	**0.036**
Overall haplotype analysis				**0.0296**	

[a]Permutated *P*-value calculated with the Haploview program, correcting for multiple testing. Significant *P*-values are marked in bold, and *P*-values between 0.1 and 0.05 are in italics.

Table 4. Independent effect test in the Belgian–Dutch and the French population

SNP number	Base substitution	Amino acid substitution	*P*-value	
			B–D population	French population
2	29T → C	L10P	0.840	*0.092*
3	74G → C	R25P	0.689	0.171
5	788C → T	T263I	**0.011**	**0.0026**
7	861-20C → T		*0.082*	0.406

Significant *P*-values are marked in bold, and *P*-values between 0.1 and 0.05 are in italics.

Table 5. Case–control association analysis of TGF-β1 SNPs with otosclerosis in the French population

SNP number	Amino-acid substitution	Allele frequency (no.)			Odds ratio	Nominal *P*-value	Permutated *P*-value[a]
		Allele	Case	Control			
2	L10P	T	0.63 (561)	0.56 (557)	1.260 (1.051–1.510)	**0.012**	*0.052*
		C	0.37 (347)	0.44 (437)			
3	R25P	G	0.938 (842)	0.947 (941)	0.818 (0.558–1.198)	0.301	0.740
		C	0.062 (58)	0.053 (53)			
5	T263I	C	0.985 (900)	0.957 (951)	0.334 (0.180–0.620)	**0.00019**	**0.0014**
		T	0.015 (14)	0.043 (43)			
7		C	0.73 (659)	0.74 (736)	1.101 (0.897–1.351)	0.359	0.859
		T	0.27 (253)	0.26 (258)			

[a]Permutated *P*-value calculated with the Haploview program, correcting for multiple testing. Significant *P*-values are marked in bold, and *P*-values between 0.1 and 0.05 are in italics. 95% CI is given within brackets.

the activity of the Ile263 variant was consistently higher than the Thr263 variant in each individual transfection experiment.

The analysis showed a significant difference between the Thr263 variant and the Ile263 variant with a *P*-value of 1.6×10^{-6} (Fig. 3). The average relative luciferase activity of the Ile263 variant was 0.78 units or 21.2% higher when compared with the average luciferase activity of the Thr263 variant after correcting for the random variation between individual transfection experiments using a two-way Anova. This result supports a protective nature for the Ile263 variant.

DISCUSSION

We have completed a case–control association study of TGF-β1 variants with otosclerosis in two independent populations. TGF-β1 is a multi-functional growth factor that regulates a broad range of biological processes. Related to otosclerosis, its most important function is its role in the chondrogenesis of the otic capsule. Moreover, the expression of TGF-β1 in adult human otosclerotic stapedial bone suggests a role in the bone turnover of the otic capsule (36). Therefore, we and other researchers believe that TGF-β1 is a good candidate for involvement in the pathogenesis of otosclerosis (33–40).

Consistent with this hypothesis, our SNP analysis in the B–D population and the French population revealed that the rare genotype (allele coding for Ile) of the associated SNP T263I is more frequent in the control population. This points to a protective role of I263 in the development of otosclerosis. The results of the association testing being replicated in an independent population, and in addition surviving two different multiple testing corrections (Bonferroni and permutation test), strongly suggest that the association of otosclerosis with TGF-β1 is real and that TGF-β1 has an influence on

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Table 6. Haplotype analysis of the possible functional *TGF-β1* SNPs with otosclerosis in the French population

Haplotypes		Case frequency	Control frequency	Nominal	Permutated *P*-value[a]
In base pairs	In amino acid				
TGCC	L10 R25 T263 861-20C	0.435	0.402	0.426	0.876
CGCC	P10 R 25 T263 861-20C	0.221	0.251	0.179	0.688
TGCT	L10 R25 T263 861-20T	0.193	0.159	**0.028**	0.185
CGCT	P10 R25 T263 861-20T	0.067	0.092	0.242	0.784
CCCC	P10 P25 T263 861-20C	0.057	0.047	0.252	0.806
CGTC	P10 R25 I263 861-20C	0.020	0.041	**0.00031**	**0.0019**
Overall haplotype analysis				**0.00144**	

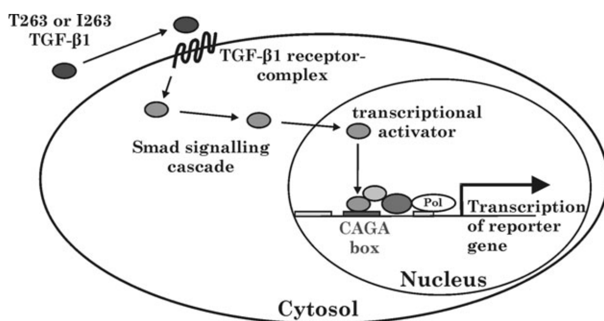

Figure 2. The principles of the TGF-β1 reporter assay. TGF-β1 containing the T263 or I263 variant will bind to its receptor, and through the Smad signaling pathway it will activate the transcription of the reporter gene. The CAGA box is the TGF-β responsive promoter element (48). Pol, polymerase.

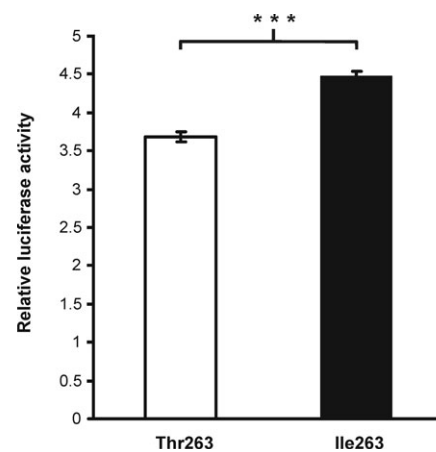

Figure 3. Effect of Thr263 and protective (Ile263) TGF-β1 constructs. Lysates of HEK293T cells transiently expressed with a TGF-β responsive reporter construct, a TGF-β1 containing expression construct, and the pRL-TK vector were collected. The transfection experiments were performed 10 times in duplo. Lysates were assayed for Firefly and Renilla luciferase activity in duplo. The ratio of both activities is given to correct for transfection efficiency. ****P*-value of 1.6×10^{-6}. The error bars indicate SEM.

the susceptibility for otosclerosis. Besides T263I, other SNPs were significantly associated in the B–D and the French population. However, these SNPs did not survive the multiple testing correction and the independent effect test in WHAP showed in both populations that these other associations are probably due to LD with SNP T263I, this latter variant being the only true causal variant.

Although many diseases like osteoporosis, asthma, cancers, osteoarthritis, hypertension and non-syndromic cleft lip show association with coding SNPs in TGF-β1, no study has reported an association for the SNP T263I (49–55). The T263I variant, therefore, may confer susceptibility specifically for otosclerosis and not other complex diseases. TGF-β1 is not only involved in the susceptibility of complex diseases. Camurati-Engelman disease (CED) or Progressive Diaphyseal Dysplasia (MIM 131300) is a rare bone disorder with an autosomal dominant mode of inheritance. The disease is caused by mutations in TGF-β1, of which most of them are situated around the cystein bridges in the LAP (56). The pathology arises due to an increased TGF-β1 signaling as a result of disturbed activation or secretion of the mutant protein (57).

Our functional analysis, using the reporter assay, shows a very significant and consistent difference in luciferase activity between Ile263 and Thr263 TGF-β1, with Ile263 being the more active variant (*P*-value of 1.6×10^{-6}). We could not detect a difference in the concentration of active or total TGF-β1 in lysate or medium of the cells using ELISA (data not shown). The absence of a difference in total TGF-β1 concentration could be explained by an effect of the Ile263 variant on the activation of TGF-β1 and not on the secretion. Moreover, as the ELISA assay measures active TGF-β1 concentration in medium or lysate at a given point instead of TGF-β1 activity during the whole incubation period, as the luciferase assay does, subtle differences between both variants on active TGF-β1 concentration might easily remain undetected. This is in line with previous observations for monogenic TGF-β1 mutations leading to CED, where a 35-fold increase in TGF-β1 activity (the luciferase assay) correlates with only a 2-fold increase in concentration of active TGF-β1 (ELISA) (57).

There are several possible explanations for why the T263I polymorphism may change TGF-β1 activation. The prediction program PROSITE (58,59) proposes that amino acid Thr263 is a Casein kinase II phosphorylation site. When Thr is changed into Ile, this site is not annotated anymore. Casein kinase II is a protein serine/threonine kinase whose activity is independent of cyclic nucleotides and calcium. Although we do not have experimental evidence for this, it is possible that the loss of this phosphorylation site affects TGF-β1 activation. Alternatively, changes in this amino acid could lead to a more efficient cleavage of the mature peptide from the LAP, either

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through a direct effect, this site is only 15 amino acids away from the cleavage site, or an indirect effect (through a conformation change of the LAP).

The reason why a more active form of TGF-β1 could be protective against otosclerosis is at present unclear, but we propose the following hypothesis: Active TGF-β1 inhibits osteoclast differentiation and activation through the osteoblasts and stimulates osteoblast chemotaxis, proliferation and differentiation (60–63). The more active protective variant (Ile263) results in the production of more active TGF-β1; this is expected to inhibit osteoclast differentiation and activation. Inhibiting osteoclast activity may be important in preventing the first otospongiotic phase of otosclerosis (64) during which bone is resorbed by special osteoclasts that present CD51/61 antigens on their cell surface (65). Consequently, this would be a means to keep bone remodeling of the otic capsule under control.

In summary, in this study we have shown that TGF-β1 influences otosclerosis susceptibility in a B–D and a French population and that the SNP T263I is most likely the causal variant. Collection of additional patient sets is needed to determine whether this association is true for other otosclerosis populations. As TGF-β1 is involved in numerous pathways, it will be difficult to completely understand the effect of TGF-β1 on the development of otosclerosis only by studying TGF-β1, and additional association studies analyzing other candidate genes involved in the same pathway as TGF-β1 are required. Alternatively, whole genome association studies using high-density micro arrays would be very helpful in identifying pathways that can explain not only the effect of TGF-β1, but also the development and pathogenesis of otosclerosis in a more general way. With that knowledge, the development of prevention strategies or therapies may be possible. While we recognize that stapes surgery is very effective and in most cases restores hearing, treatment based on biological insights may have an important impact as a cheaper, safer and more easily offered option to prevent the development of hearing loss. When provided as post-surgical therapy, medical treatment may prevent disease recurrence and the need for a revision surgery or even prevent progression to inoperable sensorineural hearing loss, which ultimately affects approximately 10% of persons with otosclerosis (66,67).

MATERIALS AND METHODS

Clinical diagnosis

The selection of the otosclerosis patients was performed by audiologists who are expert in otosclerosis at participating centers in Antwerp, Ghent, Nijmegen and Colombiers. We only included patients for which otosclerosis was surgically confirmed or for which stapes surgery was recommended by the participating center.

Pure-tone audiometry was performed on all patients with air conduction at 125, 250, 500, 1000, 2000, 4000 and 8000 Hz, and bone conduction at 250, 500, 1000, 2000 and 4000 Hz. Otoscopy and tympanoscopy were performed to rule out outer or middle ear pathology, respectively, and stapedial reflex decay was measured to assess the mobility of the stapes.

Belgian–Dutch case–control study group

A total of 632 unrelated otosclerosis patients of Belgian or Dutch origin were ascertained through the Department of Otolaryngology of four different hospitals: University Hospital of Antwerp, St Augustinus Hospital in Antwerp, University Hospital of Ghent for the Belgian patients and the University Medical Center St Radboud in Nijmegen for the Dutch patients.

From a DNA repository at the Department of Medical Genetics in Antwerp, 632 controls were selected. Every patient was matched one to one with a control sample for ethnicity, age (within ± 1 year) and sex.

French case–control study group

In total, 457 French otosclerosis patients were collected through the Jean Causse Ear Clinic in Colombiers, France. The Laboratoire de Genetique Moleculaire et chromosomique CHU, IURC Montpellier in France provided 497 control samples. Patients and controls were matched for ethnicity.

Single nucleotide polymorphism selection

SNPs were selected in TGF-β1 based on their presence in a number of association studies (49,50), and their possible functional effect (Table 1). To fully cover the gene, additional SNPs were selected based on the HAPMAP phase II (release 20). TagSNPs were selected using the aggressive tagging method implemented in the program Tagger (41) with the Haploview software (68) (minor allele frequency >0.05 and $r^2 > 0.8$).

Genotyping

Blood samples from study participants were obtained after informed consent. Genomic DNA was isolated by standard techniques.

Assay designs and SNP analyses were performed by K-Biosciences (Cambridge, UK; www.kbioscience.co.uk) using their system of competitive allele-specific PCR system (KASPar). Hidden duplicates were added as a quality control of the analysis.

Association analysis

We tested all SNPs for deviation from the HWE in the controls using Transposer V1-0 software (69). None of the SNPs showed a significant deviation from HWE in any of the two control populations. A cut-off P-value of 0.001 was taken.

Association between our markers and the disease status was tested using stepwise backward logistic regression, allowing for sex effects and sex–genotype interaction. Effect modification or sex–SNP interaction was tested in a full factorial logistic regression model including the main effects of the SNP genotype, sex and the interaction term between them. The significance of the interaction term indicates whether the effect of the SNP is different between males and females. When this was significant, we tested for association between the SNP and affection status in males and females

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separately. Otherwise, we tested for significance of the SNP in a univariate logistic regression, using the LRT (70) which is asymptotically equivalent to the Cochran–Armitage trend test (ATT). Allelic frequencies and allele-specific ORs were calculated assuming an additive model.

The common OR was estimated using the Mantel–Haenszel estimator. Conditional independence was tested using the Cochran–Mantel–Haenszel test. Homogeneity of the OR was tested by the Breslow-Day test. All analyses were conducted with the statistical programs R 2.3.1 and SPSS 12.0.1 for Windows.

Correcting for multiple testing was performed with both the Bonferroni correction and the permutations test. For the permutation test, genotypes or haplotypes were randomly permuted for 10^6 iterations to generate the distribution of *P*-values under the null hypothesis, given the LD pattern of the SNPs. This corrects for multiple hypothesis tests on SNPs in LD. This test is implemented in the Haploview program (68).

For the haplotype analysis the WHAP program (43) was used. WHAP directly calculates likelihood estimates, likelihood ratios and *P*-values taking into account the loss of information due to haplotype phase uncertainty and missing genotypes. Association between the inferred haplotypes and otosclerosis was tested using an omnibus test.

To test which of the associated SNPs was causal and which were significantly associated by LD, the independent effect test was used. Hereby, the significance of each SNP is tested, fixing the genotype of the surrounding SNPs.

Cell culture condition

HEK293T, human embryonic kidney cells, were grown in Dulbecco's modified Eagle's medium with 4500 mg/l glucose, supplemented with fetal calf serum (FCS) (10% v/v), penicillin (100 units/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM). Medium and supplements were from Invitrogen (Paisley, UK).

Construction of expression vectors and transfection

A pcDNA3 plasmid (Invitrogen) containing the full-length TGF-β1 (Thr263) (57) was used. The variant Ile263 was introduced in this TGF-β1 construct by site-directed mutagenesis (Quickchange site-directed mutagenesis kit, Stratagene, La Jolla, USA). The complete sequence of the TGF-β1 insert was validated by sequencing analysis for both the Thr263 and the Ile263 TGF-β1 producing constructs. For transient expression of the respective gene products, the constructs were transfected into HEK293T cells, using the transfection reagent Fugene6 (Roche Diagnostics Belgium, Vilvoorde, Belgium). Each transfection was performed in duplicate and repeated 10 times. HEK293T cells were transfected at 60–80% confluency with 9 μl of Fugene 6, 10 ng of pRL-TK (Promega, Madison, USA), 1.5 μg of the pcDNA3 construct and 1.5 μg of the TGF-β responsive reporter construct (48) in a total volume of 2 ml for subsequent use of media and/or lysates in reporter assays and ELISAs. After 24 h of incubation, the medium was replaced with 2 ml of serum-free Opti-MEM I (Invitrogen) to avoid the influence of TGF-β1 contained in the FCS on our measurements. After another 24 h incubation of the transfected cells, the supernatants were collected. The cells were lysed with 500 μl of passive lysis buffer (Promega, Madison, USA). Media and lysates were aliquoted and stored at −80°C.

Reporter assay

A TGF-β responsive reporter construct was provided by Dr Dennler (48). Cotransfection of the pRL-TK vector, containing the herpes simplex virus-thymidine kinase (HSV-TK) promoter region upstream of the Renilla luciferase gene, allowed calibration of the Firefly luciferase activity against the Renilla luciferase activity, providing normalization of transfection efficiency. Cell lysates were assayed for Firefly and Renilla luciferase activity using the dual-luciferase reporter assay system (Promega), following the manufacturer's instructions. Briefly, 20 μl of the lysate was transferred to a 1.5 ml microfuge tube. Fifty microlitres of luciferase assay reagent II was added to the lysate, and Firefly luciferase activity was measured for a period of 10 s. After adding 50 μl of Stop&Glo reagent, Renilla luciferase activity was measured, again for a period of 10 s. This process was automatically done by the luminometer GloMax 20/20n (Promega). The ratios of both activities were determined.

Ten transfection experiments of both Thr263 and Ile263 were performed in duplo. The significance of the Ile263 effect was tested with a two-way Anova.

During every transfection experiment, a positive control experiment was performed to validate the assay. The control vectors were TGF-β1 producing pcDNA3 vectors containing a Camurati-Engelman mutation (either R218C or LLL12-13 insertion). The reporter assay results obtained with these vectors were consistent with the published results (data not shown) (57).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

We thank all the otosclerosis patients and the participants whose contribution made this work possible. This work was supported by the European Commission (FP6 Integrated project EuroHear LSHG-CT-20054-512063), the NIH (grant R01DC05218), the FWO (grant G.0138.07) and the University of Antwerp (TOP grant). M.T. holds a pre-doctoral research position with the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen). I.S. holds a predoctoral research position and K.J., K.V.D.B. holds a post-doctoral position with the 'Fonds voor Wetenschappelijk Onderzoek Vlaanderen' (FWO).

Conflict of Interest statement. None declared.

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