Case–Control Genotyping of the c.788C>T Variant of Transforming Growth Factor–Beta 1 Gene in Otosclerosis in the South Indian Population

Deepa Kale1, Santhanam Rekha1, Sigamani Vinoth2, Ravi Ramalingam3, Madasamy Parani1

1Genomics Laboratory, Department of Genetic Engineering, SRM Institute of Science and Technology, Kattankulathur, Tamil Nadu, India
2University of Tennessee Health Science Center, Memphis, Tennessee, TN, USA
3KKR ENT Hospital and Research Institute, Chennai, Tamil Nadu, India

ORCID IDs of the authors: S.V. 0000-0002-7545-3907; M.P. 0000-0002-2265-1715.

Cite this article as: Kale D, Rekha S, Vinoth S, Ramalingam R, Parani M. Case–control genotyping of the c.788C>T variant of transforming growth factor-beta 1 gene in otosclerosis in the south indian population. J Int Adv Otol. 2022;18(2):112-117.

BACKGROUND: Otosclerosis is a common conductive hearing loss resulting from abnormal bone metabolism. The c.788C>T variant in the transforming growth factor-beta 1 gene is associated with otosclerosis in all studied populations, except the Indian population. In this study, we predicted the functional effects of reported variants in transforming growth factor-beta 1 and analyzed the c.788C>T variant in a case–control cohort from India and in the genomes present in public databases.

METHODS: Clinically confirmed otosclerosis cases (n = 120) and controls (n = 120) were recruited and genotyped by polymerase chain reaction-restriction fragment length polymorphism and DNA sequencing. In addition, Ensembl 1000 Genome, Ensembl NHLBI Exome, GnomAD, and Genome Asia 100K human genome databases were analyzed for allele frequency.

RESULTS: Among the 3 variants studied, a significant functional effect was observed only for the c.788C>T variant. This variant was found in 1 case but absent in all others and controls. Odds ratio, 95% CI, and P-value under the dominant model were 1.00, 0.0197-50.8116, and 1.00, respectively. Analysis of genomic databases showed a frequency of 0-11.21% and 0-1.25% for the c.788C>T variant and the individuals homozygous for this variant, respectively.

CONCLUSION: We did not find any genetic association between the c.788C>T variant and otosclerosis in the South Indian population; however, it was not monomorphic as had previously been reported from the Odisha population of Eastern India. Moreover, contrary to an earlier report that the c.788C>T variant was never found in a homozygous condition, homozygous individuals were found in the European, Asian, Latin American, and Ashkenazi Jews populations.

KEYWORDS: c.788C>T variant, case–control cohorts, Otosclerosis, OTSC, rs1800472, TGFB1

INTRODUCTION
Otosclerosis (OTSC) is characterized by abnormal remodeling of the otic capsule, leading to reduced resorption via the osteoclast, as well as the deposition of a bony layer via the osteoblast. The incidence of OTSC was found to be in the range of 0.03-2.5% in the Caucasian, Tunisians, American Indians, and Japanese populations. So far, the highest incidence of reported OTSC is 10-17% in the Todas, a small tribal group from Tamil Nadu, India. Otosclerosis is considered to have a complex etiology and consists of both non-familial and familial forms. In non-familial cases, external factors such as viral infection and increased estrogen levels are associated with OTSC. In familial cases, OTSC is mainly an autosomal dominant disease with reduced penetrance and variable expressivity. The age of onset is variable with age- and pregnancy-related increases in severity. It was reported that the age of onset of OTSC was delayed in successive generations.

So far, ten loci associated with OTSC (OTSC1-10) have been identified. Genetic association studies identified etiologic polymorphisms in transforming growth factor–beta 1 (TGFB1), collagen type I alpha 1 chain (COL1A1), reelin (RELN), bone morphogenetic protein 2 (BMP2), bone morphogenetic protein 4 (BMP4), angiotensin-converting enzyme insertion/deletion (ACE I/D), angiotensinogen,
found to be monomorphic.14 Therefore, in this study, (i) 3 variants of
TGFB1 were genotyped in a case-control OTSC cohort consisting of
104 males and 74 females, who were recruited from the KKR ENT
Hospital and Research Institute, Chennai, Tamil Nadu, India, from
2016 to 2019. Ethical approval for this study was granted by the
Institutional Ethical Committee of the SRM Medical College Hospital
and Research Centre, SRM Institute of Science and Technology (Ethics
Clearance Number: 796/IEC/2015). Informed written consent was
obtained from all the participants of the study before initiating the
process. Diagnosis of OTSC was based on audiological analysis, pure
tone audiometry, tympanometry, and impedance testing. Pure tone
audiometry was performed for air and bone conduction of threshold
values at 0.125 kHz, 0.25 kHz, 0.5 kHz, 1 kHz, 2 kHz, 3 kHz, 4 kHz, 6 kHz,
and 8 kHz in both ears. Pure tone audiometry, tympanometry, and
imittance audiometry were evaluated under expert supervision. A
total of 120 control individuals over 50 years of age (65 males and 55
females) were selected at random with normal hearing sensitivity in
addition to no history of hearing impairment in their families.

Genotyping of the c.788C>T Variant in the TGFB1 Gene
Blood samples (2 mL) from cases and controls were collected under
erelative conditions in potassium-ethylenediaminetetraacetic acid
vacuutainers. Isolation of genomic DNA was done using the modi-
fied Miller method.13 Presence of the c.788C>T variant in the TGFB1
gene results in the gain of a restriction site for the BstCI restriction
enzyme (5’GGATGN and 5’NNCATCC). Primers binding at unequal
distances from this BstCI restriction site were designed to amplify
an 840-bp fragment, which included 2 other BstCI sites apart from
the mutational site. The polymerase chain reaction-restriction frag-
dent length polymorphism (PCR-RFLP) method devised to screen
for the c.788C>T variant based on these restriction sites are shown
in Figure 1. Polymerase chain reaction was performed with 100 ng
of genomic DNA in 20 µL of the reaction consisting of 2 units of Taq
DNA polymerase enzyme (GenetBio, South Korea), 2 µL 10× buffer,
1 µL 10 mM dNTPs (New England Biolabs, USA) and 5 pmol prim-
ers (Eurofins, India). Samples were initially denatured at 94°C for 5
minutes followed by 35 cycles of denaturation at 94°C for 1 minute,
annealing at 60°C for 30 seconds, extension at 72°C for 1 minute, and
a final extension at 72°C for 5 min in a thermal cycler (Eppendorf,
Germany). Amplified PCR products were restriction digested without
purification. Restriction digestion was set up with a 20 µL reaction
consisting of 10 µL PCR product, 2 µL 10× buffer, and 2 units BstCI
restriction enzyme (Thermo Fisher Scientific, USA) overnight at 55°C.
Restriction digested PCR products (10 µL) were electrophoresed in
2% agarose gels and stained with ethidium bromide. Samples, which
were positive for the c.788C>T variant in PCR-RFLP, were subjected
to Sanger sequencing with a DNA sequencer (Thermo Fisher Scientific).

Statistical Analysis
Hardy–Weinberg equilibrium (HWE) was tested with the χ² test.
MEDCALC software (https://www.medcalc.org) was used to calculate
the odds ratio (OR), 95% CI, and the P-value to evaluate the frequency
of the c.788C>T variant in the study population. The genetic associa-
tion between the variant and OTSC was considered statistically
significant at a P-value of less than .05.
Analysis of Genome and Exome Data

Analysis of the frequency of the c.788C>T allele in populations of different ancestry was obtained from the human whole genome and exome databases, such as Ensembl 1000 Genome, Ensembl NHLBI Exome database (https://evs.gs.washington.edu/EVS/), GnomAD (https://gnomad.broadinstitute.org/), and Genome Asia 100K (https://browser.genomeasia100k.org/)

RESULTS

In silico analysis of c.74G>C, c.788C>T, and c.29C>T nonsynonymous variants in TGFB1 predicted that the c.788C>T variant, resulting in T263I amino acid change, was possibly “damaged” by Polymorphism Phenotyping (PolyPhen), “diseased” by Mutation Taster and SNPs and Gene Ontology (SNPs&GO), and “conserved” by GERP. Detailed output of in silico analysis, using 11 bioinformatics tools and corresponding inference for all 3 variants of the TGFB1, are shown in Table 1. Prediction of the effects of the c.788C>T variant on structural and functional alteration by MutPred yielded a score of 0.367. I-Mutant analysis showed that the TGFB1 protein with the c.788C>T variant (T263I) was more stable than the wild type (DDG; 0.17). The HOPE tool predicted that isoleucine residue in the 263rd position was larger than threonine in size, and rendered the protein more hydrophobic than the wild type.

Polymerase chain reaction amplification of a region in the TGFB1 gene flanking the site of the c.788C>T variant using the primers designed in the present study yielded an 840-bp fragment. This DNA fragment was sequenced and confirmed to be derived from the expected region of the TGFB1 gene. In PCR-RFLP, 3 (464, 253, and 123bp), 4 (373, 253, 123, and 91bp), and 5 (464, 373, 253, 123, and 91bp) DNA fragments were expected from the homozygous wild type, homozygous variant, and heterozygous individuals, respectively, as described in Figure 2. In our analysis, the 91-bp fragment was not visible; however, the unique 373-bp fragment was sufficient to identify the variant allele. When the PCR-RFLP profiles were used for genotyping, 120 clinically confirmed OTSC cases and 120 controls, only 1 case was found to have the c.788C>T variant allele in a heterozygous condition (Figure 3). DNA sequencing of the PCR products confirmed the presence of heterozygous c.788C>T variant allele in this case (Figure 4). All other cases and controls had the wild type allele in a homozygous condition.

Table 1. Analysis of the Functional Effect of c.74G>C (R25P), c.788C>T (T263I), and c.29C>T (P10L) Variants of TGFB1 Gene on TGFB1 Protein Using In Silico Bioinformatics Tools

| Name of the In Silico Tool | c.74G>C (R25P) | c.788C>T (T263I) | c.29C>T (P10L) |
|----------------------------|----------------|------------------|----------------|
| rs1800471 Value            |                |                  |                |
| rs1800472 Inference        |                |                  |                |
| rs1800473 Value            |                |                  |                |
| rs1800474 Inference        |                |                  |                |
| SIFT Value                 | 0.22           | 0.38             | 0.72           |
| PolyPhen Inference         | Tolerated      | Tolerated        | Tolerated      |
| Mutation Taster Value      | 0.141          | 0.729            | 0.0           |
| Mutation Taster Inference  | Benign         | Damaging         | Benign         |
| PROVEAN Value              | 0.631          | 0.81             | 0.38           |
| PROVEAN Inference          | Diseased       | Diseased         | Neutral        |
| FATHMM Value               | −0.5           | 1.09             | 0.34           |
| FATHMM Inference           | Tolerated      | Neutral          | Neutral        |
| CONDEL Value               | 1.6            | −0.7             | 1.73           |
| CONDEL Inference           | Tolerated      | Tolerated        | Tolerated      |
| SNPs&GO Value              | 0.054          | 0.288            | 0.002          |
| SNPs&GO Inference          | Neutral        | Neutral          | Neutral        |
| PhD-SNP Value              | 0.389          | 0.509            | 0.090          |
| PhD-SNP Inference          | Neutral        | Diseased         | Neutral        |
| PANTHER Value              | 0.717          | 0.19             | 0.154          |
| PANTHER Inference          | Diseased       | Neutral          | Neutral        |
| CADD Value                 | 0.478          | 0.17             | 0.266          |
| CADD Inference             | Neutral        | Neutral          | Neutral        |
| GERP Value                 | 22.2           | 23.9             | 21.2           |
| GERP Inference             | Benign         | Benign           | Benign         |
| GERPE Value                | −1.19          | 2.88             | 0.52           |
| GERPE Inference            | Variable       | Conserved        | Conserved      |
**Genetic Model**

PCR is undigested PCR product. PCR, polymerase chain reaction.

4 fragments (464 bp, 373 bp, 253 bp, and 123 bp) in 2% agarose gel. The 91-bp fragment was not visible due to its smaller size. M is a 100-bp DNA marker and restriction enzyme (5′-GGATGNN and 5′-NNCATCC). Homozygous wild type (+/+), heterozygote (+/−), and homozygous variant (−/−) to be obtained by restriction digestion of the PCR product with BstCI restriction enzyme. PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism.

### Table 2. Statistical Analysis of Genetic Association Between c.788C>T Variant and Otosclerosis Using Different Genetic Models.

| Genetic Model | Genetic Comparison | Odds Ratio (95% CI) | P     |
|---------------|--------------------|---------------------|-------|
| Allelic       | T vs. C            | 3.0125 (0.1221-74.3247) | .5001 |
| Genotypic     | CT vs. CC          | 3.0251 (0.1220 to 75.0083) | .4992 |
| Genotypic     | TT vs. CC          | 1.0084 (0.0198 to 51.2385) | .9967 |
| Dominant      | TT vs. CC+CT       | 1.00 (0.0197 to 50.8116) | 1.00  |
| Recessive     | TT+CT vs. CC       | 3.0251 (0.1220 to 75.0083) | .4992 |

Statistical analysis of the genotyping data showed that the c.788C>T variant did not deviate from the HWE. Under the dominant model, the c.788C>T variant had an OR of 1.00 with a 95% CI of 0.0197-50.8116, and a P-value of 1.00. These results showed that the c.788C>T variant was not associated with OTSC. Results of statistical analysis of the genetic association between the c.788C>T variant and OTSC, assuming allelic, genotypic, dominant, and recessive models are shown in Table 2. Further, the OR has been calculated considering 15 308 South Asian population from GnomAD database as control samples along with 120 case-control cohort strengthens the fact that c.788C>T is not associated with OTSC having OR of 0.4136 with a 95% CI of 0.0578-2.9580 and P-value of .3791. Analysis of the frequency of the c.788C>T variant in different populations based on the genomic data in the public databases showed it to be polymorphic in most populations. The frequency of occurrence of the c.788C>T variant allele varied widely between 0% and 11.21% in the examined populations. This allele was predominantly present in a heterozygous condition, but a homozygous condition was observed at a much lower frequency, between 0% and 1.25% (Table 3).

### DISCUSSION

Otosclerosis is an autosomal dominant disease with variable penetrance and expressivity.10 Despite the identification of several OTSC loci, pathogenic genes and variants are not yet characterized. Among the genes, which were subjected to genetic association studies regarding OTSC, the TGFB1 is of great interest due to its involvement in bone development. Previous studies in different populations reported 3 nonsynonymous coding variants in TGFB1, c.74G>C, c.788C>T, and c.29C>T.14,15 In our bioinformatics analysis, based on the results from PolyPhen, Mutation Taster, SNPs&Go, and GERP, only the c.788C>T variant was predicted to have a significant functional effect on the TGFB1 protein. The MutPred score showed that the amino acid substitution, due to the c.788C>T variant, was not deleterious to the structure or function of the protein (threshold >0.5). In terms of protein stability, the TGFB1 protein with the c.788C>T variant was predicted to be more stable than the wild type. In fact, based on in vitro luciferase reporter assay, the TGFB1 protein with the c.788C>T variant was demonstrated to have enhanced activity than the wild type. As a consequence of this, it was hypothesized that the c.788C>T variant might play a protective role against OTSC.15

TGFB1 is a ubiquitous growth factor and multifunctional cytokine involved in bone development and homeostasis.12 Available reports show that the TGFB1 gene is highly polymorphic and has several variants that are associated with diseases, such as OTSC, non-syndromic cleft lip, asthma, cancer, osteoporosis, and osteoarthritis.10,12,13,20-23 Specifically, the c.788C>T variant of the TGFB1 gene was shown to be associated with OTSC. This variant was more frequent in the control than the case population, which led to hypothesize a protective role for this variant against OTSC; however, its protective mechanism is not clear, and the hypothesis remains to be validated.10 The c.788C>T variant in TGFB1 was associated with OTSC in all tested populations so far, except the Indian population. In a case-control study carried out in the Odisha population (Eastern India), the c.788C>T variant not only failed to show a genetic association

**Figure 2.** Diagnostic PCR-RFLP profiles from homozygous wild type (+/+), heterozygote (+/−), and homozygous variant (−/−) to be obtained by restriction digestion of the PCR product with BstCI restriction enzyme. PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism.

**Table 2.** Statistical Analysis of Genetic Association Between c.788C>T Variant and Otosclerosis Using Different Genetic Models.

**Figure 3.** DNA fragment (840 bp) flanking the site of c.788C>T variant in TGFB1 gene was amplified by PCR and genotyped by restriction digestion with BstCI restriction enzyme (5′-GGATGNN and 5′-NNCATCC). Homozygous wild type (+/+) shows 3 fragments (373 bp, 253 bp, and 123 bp) and heterozygote (+/−) shows 4 fragments (464 bp, 373 bp, 253 bp, and 123bp) in 2% agarose gel. The 91-bp fragment was not visible due to its smaller size. M is a 100-bp DNA marker and PCR is undigested PCR product. PCR, polymerase chain reaction.
with OTSC, but it was found to be monomorphic. In our study, this variant did not show a genetic association with OTSC; however, our conclusion is limited by the sample size. Contrary to the observation made in the Odisha population, the c.788C>T variant was found to be polymorphic in the South Indian population. A cursory look at the Indian genomes in Ensembl, GnomAD, and Genome Asia 100K databases showed that this variant is in fact polymorphic in the Indian population. A detailed analysis of the frequency of the c.788C>T variant in different populations showed that it is more prevalent in people with European ancestry (approximately 3%) and in Ashkenazi Jews (approximately 11.2%). Other than these populations, this variant is more prevalent in Asians, especially in South Asians (approximately 1%). In the recently built Genome Asia 100K database, the frequency of occurrence of the c.788C>T variant in the Indian population is 0.33%. These data reinforce our observation that the c.788C>T variant is polymorphic, and strongly indicates a prevalence in healthy individuals, although we did not detect it in our healthy controls, probably due to the small sample size. Moreover, our analysis of public genomic data showed the presence of the c.788C>T variant in a homozygous condition in 164 individuals comprising of South Asians, Ashkenazi Jews, Europeans, European Americans, and Latin Americans. The prevalence of the homoygous c.788C>T variant is low in all populations (0.02-0.1%), except for the Ashkenazi Jewish population (1.25%). This data established the presence of the homoygous c.788C>T variant in the populations, contrary to a previous report in which it was stated that it was never found in a homozygous condition. Why a protective allele is found in a relatively higher frequency in a heterozygous versus a homozygous condition is not clear? Otosclerosis being a heterogeneous disease involving multiple loci and complex etiology, analysis of genomic and proteomic level data from a large, diverse cohort would be required for a comprehensive understanding of this disease and associated variants.

Ethics Committee Approval: Ethical committee approval for this study was received from SRM Medical College Hospital and Research Centre, SRM Institute of Science and Technology (796/IEC/2015).

Informed Consent: Written consent was obtained from all the participants of the study before initiating the process.
Peer-review: Externally peer-reviewed.

Author Contributions: Concept – S.R, R.M, M.P; Design – S.R, R.M, M.P; Supervision - M.P; Resources – S.R, R.M, M.P; Materials – S.R, R.M, M.P; D.K; Data Collection and/or Processing – D.K., S.R., S.V; Analysis and/or Interpretation – D.K., S.R., R.M, M.P; Literature Search – D.K., S.R.; Writing Manuscript – D.K.; Critical Review – S.R, M.P.

Acknowledgments: The authors would like to thank the SRM Institute of Science and Technology for providing the well-equipped laboratory facility, and KKR ENT hospital for assisting in sample collection.

Declaration of Interests: The authors have no conflict of interest to declare.

Funding: This study was funded by the SRM-DBT Partnership Platform for Contemporary Research Services and Skill Development in Advanced Life Sciences Technologies (No. BT/PR12987/INF/22/205/2015).

REFERENCES
1. Declau F, van Spaendonck MV, Timmermans JP, et al. Prevalence of histologic otosclerosis: an unbiased temporal bone study in Caucasians. Adv Otorhinolaryngol. 2007;65(6-16. [CrossRef]
2. Ben Arab S, Besbes G, Hachicha S. Otosclerosis in populations living in northern Tunisia: epidemiology and etiology. Ann Otolaryngol Chir Cervicofac. 2001;118(1):19-25.
3. Altman F, Glasgold A, Macduff JP. The incidence of otosclerosis as related to race and sex. Ann Otol Rhinol Laryngol. 2015;76(2):377-392. [CrossRef]
4. Ohthani I, Baba Y, Suzuki T, Suzuki C, Kano M, Dek MRC. Why is otosclerosis of low prevalence in Japanese? Otol Neurotol. 2003;24(3):377-381. [CrossRef]
5. Kapur YP, Patt AJ. Otosclerosis in South India. Acta Otolaryngol. 1966;61(4):353-360. [CrossRef]
6. Kameswaran S, Kumar PV, Jeyapaul JJ, Manoharan S. Audiological and haematological studies on the Todas of Nilgiris. J Laryngol Otol. 1976;90(4):325-333. [CrossRef]
7. Niedermeyer HP, Häusler R, Schwab D, Neuner NT, Busch R, Arnold W. Evidence of increased average age of patients with otosclerosis. Adv Otorhinolaryngol. 2007;65:17-24. [CrossRef]
8. Markou K, Goudakos J. An overview of the etiology of otosclerosis. Eur Arch Otorhinolaryngol. 2009;266(1):25-35. [CrossRef]
9. Rekha S, Ramalingam R, Parani M. Pedigree analysis and audiological investigations of otosclerosis: an extended family based study. J Audiol Otol. 2018;22(4):223-228. [CrossRef]
10. Rudic M, Keogh I, Wagner R, et al. The pathophysiology of otosclerosis: review of current research. Hear Res. 2015;330(A):51-56. [CrossRef]