The Journal of International Advanced Otology

J Int Adv Otol 2022; 18(2): 112–117 • DOI: 10.5152/iao.2022.21170

Original Article

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

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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.[1-4] So far, the highest incidence of reported OTSC is 10-17% in the Todas, a small tribal group from Tamil Nadu, India.[5,6] 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.[7] In familial cases, OTSC is mainly an autosomal dominant disease with reduced penetrance and variable expressivity.[8] 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.[9]

So far, ten loci associated with OTSC (OTSC1-10) have been identified.[10] 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,

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Received: January 7, 2021 • **Accepted:** June 3, 2021
Available online at www.advancedotology.org

and osteoprotegerin from different populations.[10,11] Among them, *TGFB1,* which codes for the most abundant growth factor in human bone, TGFB1, is likely to play a role in the development of the otic capsule. TGFB1 is a multifunctional cytokine produced by osteoblasts, which regulates skeletal development and homeostasis.[12] The release of TGFB1 reduces bone resorption by causing a decrease in osteoclast activity. Previous studies have linked the contribution of *TGFB1* to the pathogenicity of OTSC, although the exact mechanism is still unknown.[13,14]

Transforming growth factor-beta 1 gene was shown to be associated with OTSC in Belgium-Dutch and French populations, and the c.788C>T variant in *TGFB1,* which leads to T263I amino acid substitution, was reported to play a protective role against OTSC.[15] In a replicative study, a significant association between the c.788C>T variant and OTSC was established in the Tunisian population.[16] When 13 single nucleotide polymorphisms (SNPs), which showed a significant association with OTSC in other populations, were tested in 153/300 cases/controls from a Hungarian population, only the c.788C>T variant was associated with the disease.[17] When 8 SNPs from *RELN, BMP2, COL1A1, FGF2, PPP2R5B,* and *TGFB1* were genotyped in a case–control OTSC cohort (n = 748) from the United Kingdom, c.788C>T was found to be 1 of the 2 SNPs strongly associated with OTSC.[18] However, in a study conducted with 457/497 cases/controls from the Odisha population of Eastern India, the c.788C>T variant not only lacked genetic association with OTSC but it was also found to be monomorphic.[14] Therefore, in this study, (i) 3 variants of *TGFB1* gene, reported from different populations, were subjected to in silico analysis to predict their functional effects on the TGFB1 protein, (ii) prevalence of the c.788C>T variant in the South Indian population was analyzed by genotyping a case–control OTSC cohort (n = 240), and (iii) analysis of the frequency of the c.788C>T variant in the public genome and exome databases was carried out to understand its genetics.

MATERIALS AND METHODS

The present study was conducted in the Genomics Laboratory of the Department of Genetic Engineering, SRM Institute of Science and Technology, from January 2016 to March 2020. Ethical approval was taken by the institutional ethical board. Written consent was obtained from all the participants of the study before initiating the process.

In Silico Analysis of the Variants in the *TGFB1* Gene

Three variants of the *TGFB1* gene, c.74G>C, c.788C>T, and c.29C>T, found in OTSC cases from different populations (14-18), were subjected to in silico analysis to predict their functional effects on the encoded protein using SIFT, PolyPhen (https://asia.ensembl.org/), Mutation Taste, PROVEAN, FATHMM, CONDEL, SNPs&GO, PhD-SNP, PANTHER, CADD, and Genomic Evolutionary Rate Profiling (GERP) (https://asia.ensembl.org/). MutPred was used to predict if the amino acid substitution would be disease-associated or neutral (http://mutpred.mutdb.org/index.html#qform). I-Mutant 2.0 was used to predict the effect of the variants on protein stability (https://folding.biofold.org/cgi-bin/i-mutant2.0.cgi). Wild type and mutant protein sequences were submitted to predict protein stability, while the conditions were set at a temperature of 25°C and a pH of 7.0. Finally, HOPE software was used to predict the structural effect of the variants (https://www3.cmbi.umcn.nl/hope/).

Case-Control Cohort and Clinical Diagnosis

Otosclerosis cases for this study consisted of 120 unrelated patients (67 males and 53 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 immittance 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 sterile conditions in potassium-ethylenediaminetetraacetic acid vacutainers. Isolation of genomic DNA was done using the modified Miller method.[19] Presence of the c.788C>T variant in the *TGFB1* gene results in the gain of a restriction site for the *Bts*CI restriction enzyme (5′-GGATGNN and 5′-NNCATCC). Primers binding at unequal distances from this *Bts*CI restriction site were designed to amplify an 840-bp fragment, which included 2 other *Bts*CI sites apart from the mutational site. The polymerase chain reaction-restriction fragment 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 primers (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 *Bts*CI 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 χ^2 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 association between the variant and OTSC was considered statistically significant at a *P*-value of less than .05.

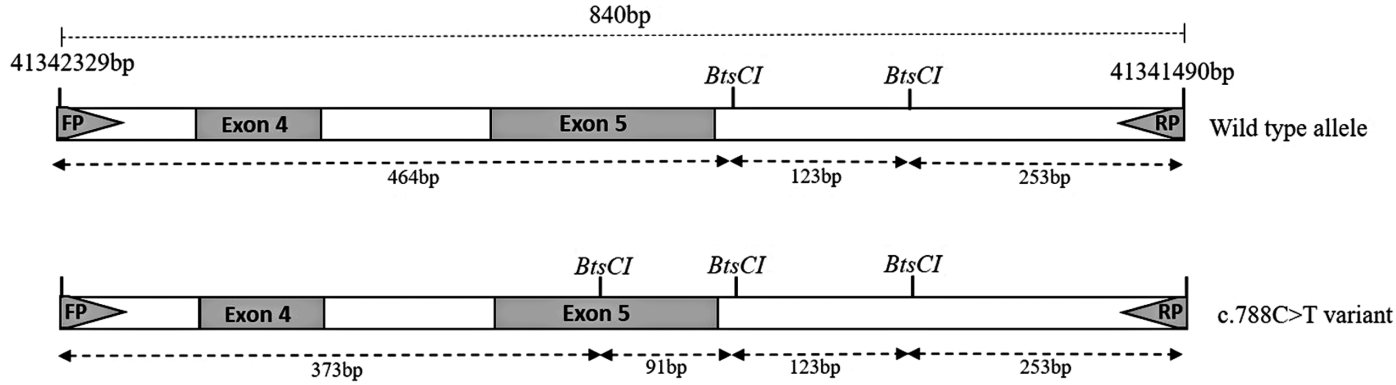

Figure 1. Schematic representation of genotyping of the c.788C>T variant in *TGFB1* gene by restriction enzyme *Bts*CI. The 840-bp DNA fragment in *TGFB1* gene to be amplified by PCR from the wild type and the c.788C>T variant allele are shown with primer binding sites with forward primer (FP: 5′-GGTTTGCTCCTTCCTTCCTC-3′) and reverse primer (RP: 5′-AAAGGAACCTGATCCCAACA-3′), exons, and *Bts*CI sites. PCR, polymerase chain reaction.

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.788 C>T (T263I), and c.29C>T (P10L) Variants of *TGFB1* Gene on TGFB1 Protein Using In Silico Bioinformatics Tools

	Nonsynonymous Variants in *TGFB1* Selected for In Silico Analysis					
	c.74G>C (R25P)		**c.788 C>T (T263I)**		**c.29C>T (P10L)**	
	rs1800471		**rs1800472**		**rs1800473**	
Name of the In Silico Tool	**Value**	**Inference**	**Value**	**Inference**	**Value**	**Inference**
SIFT	0.22	Tolerated	0.38	Tolerated	0.72	Tolerated
PolyPhen	0.141	Benign	0.729	Damaging	0	Benign
Mutation Taster	0.631	Diseased	0.81	Diseased	0.38	Neutral
PROVEAN	−0.5	Tolerated	1.09	Neutral	0.34	Neutral
FATHMM	1.6	Tolerated	-0.7	Tolerated	1.73	Tolerated
CONDEL	0.054	Neutral	0.288	Neutral	0.002	Neutral
SNP&GO	0.389	Neutral	0.509	Diseased	0.090	Neutral
PhD-SNP	0.717	Diseased	0.19	Neutral	0.154	Neutral
PANTHER	0.478	Neutral	0.17	Neutral	0.266	Neutral
CADD	22.2	Benign	23.9	Benign	21.2	Benign
GERP	−1.19	Variable	2.88	Conserved	0.52	Conserved

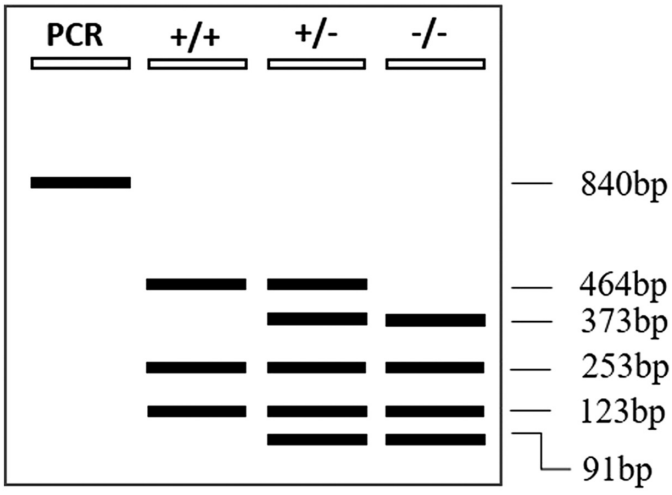

Figure 2. Diagnostic PCR-RFLP profiles from homozygous wild type (+/+), heterozygote (+/–), and homozygous variant (–/–) to be obtained by restriction digestion of the PCR product with *Bts*CI restriction enzyme. PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism.

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	1.000 (0.0197 to 50.8116)	1.000
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.[14-18] 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 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.[15,20-23] 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.[15] 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

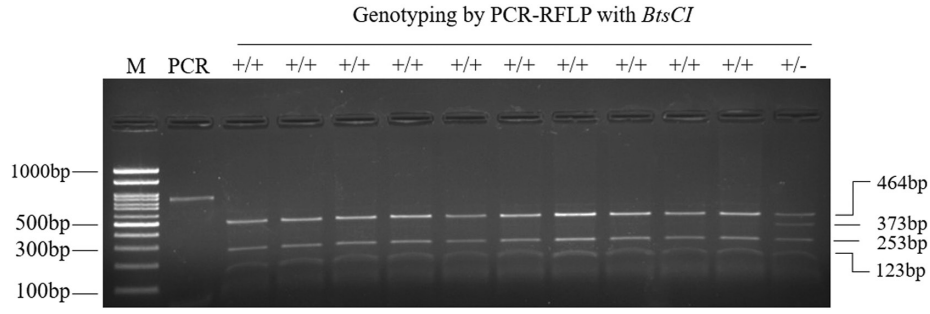

Figure 3. DNA fragment (840 bp) flanking the site of c.788C>T variant in *TGFB1* gene was amplified by PCR and genotyped by restriction digestion with *Bts*CI restriction enzyme (5′-GGATGNN and 5′-NNCATCC). Homozygous wild type (+/+) shows 3 fragments (373 bp, 253 bp, and 123 bp) and heterozygote (+/–) 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.

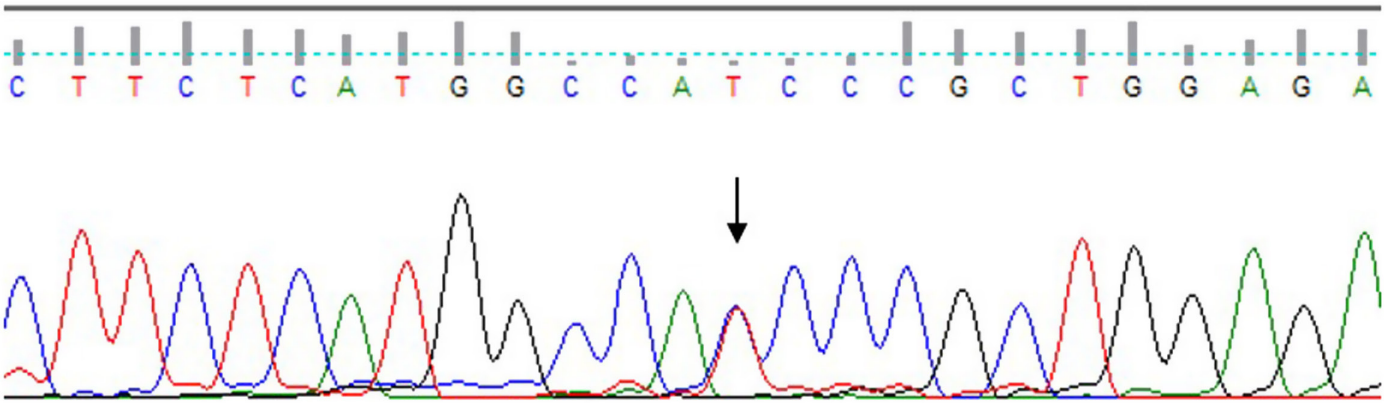

Figure 4. Chromatogram of a part of the DNA sequence from an individual heterozygous for c.788C>T variant. A double peak C and T characteristic of the heterozygous condition is indicated by an arrow.

Table 3. Frequency of Occurrence of the c.788C>T Variant of *TGFB1* Gene and the Individual Homozygous for This Variant Determined Based on the Genome and Exome Data Available in Ensembl 1000 Genome, Ensembl NHLBI Exome, GnomAD, and Genome Asia 100K Databases

Ancestry/Population	Number of c.788C>T Alleles	Total Number of Alleles Counted	Frequency of c.788C>T Allele (%)	Number of Homozygous Individuals (%)	Source
European	5043	163574	3.083	84 (0.10)	Ensembl 1000 Genome, Ensembl NHLBI Exome, GnomAD
African	112	30882	0.363	0	Ensembl 1000 Genome, Ensembl NHLBI Exome, GnomAD, Genome Asia 100K
Southeast Asian	0	692	0.000	0	Genome Asia 100K
East Asian	1	21656	0.005	0	Ensembl 1000 Genome, GnomAD, Genome Asia 100K
South Asian	325	33042	0.984	5 (0.03)	Ensembl 1000 Genome, GnomAD, Genome Asia 100K
Asian	326	55390	0.589	5 (0.02)	Ensembl 1000 Genome, GnomAD, Genome Asia 100K
Indian	4	1196	0.334	0	Genome Asia 100K
Latin American	661	35242	1.876	7 (0.04)	GnomAD
Ashkenazi Jewish	1161	10352	11.21	65 (1.25)	GnomAD

with OTSC, but it was found to be monomorphic.[14] 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,[24] 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 homozygous 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 homozygous 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.[15] 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).

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