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Rare variants in *BMP2* and *BMP4* found in otosclerosis patients reduce Smad signaling

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Abstract

Hypothesis—Genetic variation in *BMP2* and *BMP4* found in otosclerosis patients result in altered Smad signaling.

Background—Otosclerosis is a common form of adult-onset conductive hearing loss resulting from abnormal bone remodeling of the bony labyrinth that surrounds the inner ear. Both genetic and environmental factors are implicated in the disease, yet very little is known about its pathogenesis. The evidence for a genetic component has been established through family-based linkage and population-based association studies. Previously, members of the TGF- β superfamily of genes have been associated with otosclerosis.

Methods—Sequencing of *BMP2* and *BMP4* coding regions was performed to identify common and rare variation in German otosclerosis patients compared with controls. Functional analyses of rare variation in the patient cohort was done by exposing an osteosarcoma cell line to conditioned media containing either wild type or variant forms of BMP2 or BMP4 and analyzing Smad1/5/8 phosphorylation.

Results—While no significant association with common variation in these two genes was detected, there were 8 singleton variants identified in the German population. Of the four coding variants, found solely in otosclerosis patients, two – BMP4^{N150K} and BMP2^{K357-R396del} – were found to decrease Smad1/5/8 signaling.

Conclusion—Rare variants in *BMP2* and *BMP4* are not a major genetic component in the otosclerosis population. However, those with functional affect showed decreased Smad signaling. Further analysis of Smad signaling molecules should be performed to determine if these pathways in combination are a major contributor to otosclerosis, which could lead to additional treatment options for otosclerosis patients.

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Conflict of Interest

The authors declare no conflict of interest.

Introduction

One of the most common causes of conductive hearing loss in the adult population is otosclerosis, a disease of the bony labyrinth that surrounds the inner ear. In normal circumstances, the bony labyrinth undergoes very little bone remodeling after development due to the presence of inhibitory factors such as osteoprotegerin, which is secreted into the perilymph in high volumes by cells of the membranous labyrinth. These factors diffuse into the surrounding bony labyrinth through the lacunocanalicular system (1,2). This mechanism limits bone remodeling in the bony labyrinth to about 0.13% turnover per year, roughly one-one hundredth of the remodeling rate of the rest of the skeleton, which has a turnover rate of 10% per year (3).

The onset of otosclerosis is characterized by increased bone remodeling of the bony labyrinth, with a predilection for the fissula ante fenestram, a small cleft of connective tissue between the anterior portion of the oval window and the cochleariform process. Conductive hearing loss occurs when the otosclerotic focus extends posteriorly into the stapedial-vestibular joint causing fixation of the stapes. Occasionally, a faint pink-tinge known as Schwartze's sign can be seen through the tympanic membrane on the promontory, the pink color reflecting the high vascularization of the lesion.

Environmental and genetic factors are believed to contribute to otosclerosis, however despite major efforts over the last several decades, very little is known about its etiology. Amongst environmental factors, measles virus infection and hormones have been implicated; however, a direct link between these factors and otosclerosis is lacking (4–9). Evidence for a genetic component in the form of family linkage studies is robust and 10 otosclerotic loci have been mapped (*OTSC1-10*) (10–17). Paradoxically, with the exception of the *TRB* locus, which has been implicated as the causative gene in the *OTSC2* region, causal genes have not been identified at the other *OTSC* loci (18). Population-based association studies have also identified a number of associated genes (19–32). Of these association studies, two have shown association with genes in the TGF- β superfamily – *TGFB1*, *BMP2* and *BMP4*. Further evidence for the role of BMPs in the progression of otosclerosis has been provided by several protein expression studies, showing the presence of BMPs in active otosclerotic foci (33–35). However, evidence is lacking for how these proteins contribute to the pathology of otosclerosis.

In this study we sought to further characterize the role of BMPs in otosclerosis by genotyping common variants in *BMP2* and *BMP4* in a German otosclerosis population. Although we could not show association of common variants in either *BMP2* or *BMP4* with otosclerosis, by sequencing these genes we identified rare variants in the otosclerosis cohort. These variants were analyzed to determine whether different variants had similar functional effects, suggesting a common mechanism by which *BMP2* and *BMP4* play a role in the pathogenesis of otosclerosis.

Materials and Methods

Study population

The study population consisted of 265 German patients with surgically verified otosclerosis; the control population consisted of 206 German individuals matched for age, sex, and ethnicity. The mean age of both the case and control populations was 55 years (range, 18–89 years), and the female-to-male ratio was 1.6 and 1.1, respectively. DNA was isolated from blood using standard techniques. All subjects were enrolled at the University of Tübingen, Germany, and the Human Research Institutional Review Boards of the Universities of Iowa and the University Hospital Tübingen approved all procedures.

Power calculations

Power calculations were performed using the Genetic Power Calculator (<http://pngu.mgh.harvard.edu/~purcell/gpc/>). The disease allele frequency and the odds ratio (OR) of each SNP were calculated using data from a previous study (24). The relative risk (RR) of the polymorphism was estimated by the OR because the RR reliably approaches the OR when disease prevalence is 0.3%–0.4% (36). The disease frequency was set at 0.35%. Power calculations were done assuming that controls were not screened for otosclerosis and that a small percentage carries variants that contribute to disease. The disease model and the effect sizes for each SNP were based on results from our previous association study in the Belgian-Dutch and French populations (24).

Population substructure analysis

Population substructure was analyzed by genotyping the *LCT* promoter polymorphism rs4988235. This SNP varies widely across Europe, following the domestication of cattle and tolerance to lactose (37). Comparison of allele frequencies between cases and controls ruled out substructure ($p = 0.753$). The non-significant p -value shows that the cases and controls have similar genotype ratios at this locus, suggesting that genetic backgrounds are similar.

Sequencing and genotyping

Exons and flanking sequences of *BMP2* and *BMP4* were amplified by PCR, followed by Sanger sequencing. Sequencing reactions were analyzed on an Applied Biosystems 3130xl Genetic Analyzer. Chromatograms were analyzed and variants called using Sequencher v4.8 sequence analysis software. Variants were considered positive if seen in more than one sequencing run and in both sequencing directions. Genotyping fidelity was analyzed by testing for Hardy-Weinberg equilibrium; SNPs showing deviation from Hardy-Weinberg equilibrium ($p < 0.001$) were removed from the analysis.

Association testing

Since otosclerosis preferentially affects females, interaction between gender and genotype was determined for all common variants. A logistic regression model was constructed to include parameters for sex and genotype. If there was significant interaction between polymorphism and gender, males and females were analyzed separately. An additive model was built using a linear regression model assuming causative alleles have an additive effect. For this model the genotypes were coded linearly to represent the additive affect of causative alleles. In addition, a dominant/recessive model was also tested, where the dominant alleles were combined for the analysis (alleles with the minor allele frequency in the general population were considered the recessive allele). The odds ratio (OR) for each SNP was calculated with Wald 95% confidence intervals (CI). Statistical analyses of rare variants were collapsed to analyze the genes together using two-tailed Fisher's exact test.

In silico analysis of variants

To predict the pathogenesis of each rare variant, web-based software was applied. The Conseq server (<http://conseq.tau.ac.il/>) uses over 40 species to determine the conservation score (1 – least conserved to 9 – highly conserved) of each amino acid in a protein. The SIFT program (<http://sift.bii.a-star.edu.sg>) uses protein sequence homologies to predict whether an amino acid substitution will affect the function of the protein of interest (38). The PolyPhen program (<http://genetics.bwh.harvard.edu/pph/index.html>) uses sequence homology and mapping of the variant sites to known 3D structures (39).

Expression vectors

Full-length human *BMP2* and *BMP4* wild type cDNA was amplified from a human kidney cDNA library (Invitrogen) and cloned into the pcDNA3.1zeo-man expression vector (Invitrogen). Variants from the German patients were introduced into the *BMP2* or *BMP4* wild type vector using the QuikChange site-directed mutagenesis kit and following the manufacturer's protocol (Agilent).

Cell culture conditions

HEK293T cells were maintained in Dulbecco's modified Eagle's media (Life Technologies) supplemented with 10% FBS (Atlanta Biologicals), 1% L-glutamine (Invitrogen), and 1% penicillin/streptomycin (Invitrogen). MG-63 osteosarcoma cells were maintained in Eagle's minimal essential media (Life Technologies) supplemented with 10% heat-inactivated FBS (Lonza), 1% nonessential amino acids (Invitrogen), 1% sodium pyruvate (Invitrogen), and 1% penicillin/streptomycin (Invitrogen).

Transfections and conditioned media treatment of cells

For transfections, HEK293T cells were plated at a density of 3×10^6 in 10cm plates. Cells were transfected at 60–70% confluency using Xtremegene 9 transfection reagent (Roche). Each set of transfections was performed 5 times. Media was replaced with serum-free media 24 hours post transfection to remove any influence of TGF- β or BMPs from the serum, and incubated for 24 hours to condition the media with transfected *BMP2* or *BMP4*.

MG-63 cells were plated at a density of 3×10^6 in 10cm plates. MG-63 cells were grown to 80% confluence and media was replaced with serum-free media for 24 hours. MG-63 cells were treated with 4ml of conditioned media from transfected HEK293T cells for 1 hour. Each conditioned media treatment was performed in duplicate. Remaining conditioned media from the transfected HEK293T was precipitated with trichloroacetic acid. The protein pellet was dissolved in Laemmli sample buffer and stored at -20°C . HEK293T cells and MG-63 cells treated with conditioned media were lysed with Radio-Immunoprecipitation Assay (RIPA) buffer (Sigma-Aldrich) containing protease inhibitors at 4°C , cell debris pelleted, and protein containing supernatant saved and stored at -20°C .

Western blot

Cell lysates were diluted in Laemmli sample buffer containing β -mercaptoethanol and denatured at 100°C for 10 minutes. Samples were loaded on 4–15% mini-Protean tris/glycine gels (Biorad) and protein transferred to nitrocellulose. Membranes were blocked for 1 hour at room temperature in 5% milk then blotted overnight with one of the following primary antibodies: 1:500 rabbit anti-Smad1/5/8 (Cell Signaling), 1:500 rabbit anti-Smad1 (Cell Signaling), 1:500 rabbit anti-BMP4 (Abcam), or 1:500 rabbit anti-BMP2 (Abcam). After washes with 1x TBS/T, blots were incubated for 3 hours at room temperature with 1:3000 goat anti-rabbit secondary antibody (Cell Signaling). Blots were developed using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific) and exposed to film.

Results

Association testing

SNPs in *BMP2* and *BMP4* were genotyped by Sanger sequencing and tested for association by building a logistic regression model for each SNP; a likelihood ratio test (LRT) was used to test for association. The genotypes were coded linearly to assume an additive model of disease, where the heterozygous genotype has an intermediate phenotype. To analyze a

dominant/recessive model of disease the dominant alleles were combined for analysis, where the minor alleles were considered recessive. We were unable to replicate the association of the previously associated SNPs (rs3178250 in *BMP2* and rs17563 in *BMP4*) in the German population using either an additive model or dominant/recessive model of disease (Table 1).

To determine whether either gene was associated with otosclerosis (rather than specific variants), we sequenced *BMP2* and *BMP4* in cases and controls and interrogated all identified variations. Of the 16 variants identified, eight are present in dbSNP 132 (6 SNPs in *BMP2* and 2 SNPs in *BMP4*) (Table 2). None of these SNPs was associated with otosclerosis using either the additive or dominant/negative models (Table 1). There was no significant difference between males and females for all of the SNPs tested (Table 1). Population substructure is unlikely a cause for this failure, and while insufficient power is a possibility for *BMP2*, for *BMP4* our power was excellent (96%, Table 3).

Rare variant analysis

Eight rare variants were identified in the German population. Only one *BMP2* 3' UTR variant in a German control was found in 1000 Genomes data (rs183175284, European MAF: 0.003). The remaining seven variants are not present in dbSNP 135, 1000 Genomes, or the Washington Exome Server database (Table 2, Figure 1). Although these novel variants were identified in both coding and non-coding regions of both genes and in both cases and controls, the four coding variants were found only in otosclerosis patients (Fisher's exact $p = 0.1352$).

BMP2 carried two coding variants, c.314G>A resulting in a heterozygous R105Q variant and a homozygous deletion of the C-terminal portion of the protein (c.1072_1414del, p.K357-R396del, Figure 1). In *BMP4*, we identified an N150K variant that has been reported in two individuals with renal hypodysplasia (40). The fourth coding variant was a synonymous change in *BMP4* (c.76C>T, p.L26L), which is not predicted to affect splicing by *in silico* analysis and no further analysis was performed.

Possible impact of the nonsynonymous substitutions was analyzed using Conseq, SIFT, and PolyPhen. Both the *BMP2*^{R105Q} variant and the *BMP4*^{N150K} variant were predicted to have little effect on protein function (*BMP2*^{R105Q}: Conseq, 5; SIFT, tolerated, 0.08; Polyphen, benign, 0.993; *BMP4*^{N150K}: Conseq, 4; SIFT, tolerated, 0.19; Polyphen, benign, 0.863). However the deletion of the C-terminal 40 amino acids of *BMP2* was predicted to be damaging: the last 40 amino acids of *BMP2* are highly conserved, with an average Conseq score of eight.

Functional analysis of nonsynonymous rare variants

To test functional relevance, we analyzed downstream BMP signaling events focusing on phosphorylation of the receptor Smad for *BMP2/4* signaling, Smad1/5/8. The osteosarcoma cell line MG-63 is responsive to BMP signaling as demonstrated by the robust phosphorylation of Smad1/5/8 in cells treated with wild type *BMP2* and *BMP4* (Figure 2). The *BMP2*^{R105Q} variant also leads to Smad1/5/8 phosphorylation. However, neither the K357-R396del in *BMP2* and the N150K variant in *BMP4* were able to activate Smad1/5/8 in MG-63 cells (Figure 2).

Discussion

Population-based genetic association studies have revealed a number of novel candidate variants for multiple diseases however very little clinically relevant knowledge has been

gained. To bridge this gap, deep sequencing of associated candidate genes in targeted populations should be undertaken. Rare and novel coding variants can then be studied *in vitro* to identify possible functional effects in disease-relevant pathways as a first step towards developing personalized treatments.

In this study, we sought to replicate the association of *BMP2* and *BMP4* with otosclerosis, and to identify patient-specific variants of functional significance in protein signaling. Although replication of association studies in multiple populations is crucial to their validation (24), neither of the originally associated SNPs in these two genes was associated with otosclerosis in the German population we studied. Power calculations for the two variants showed that our study population is underpowered to replicate the original association with *BMP4*, but has excellent power to replicate the *BMP2* finding. However, the odds ratio we originally reported has a wide confidence interval, which may explain our negative result (41,42). We also considered differences in LD structure, which we excluded by sequencing both genes in cases and controls and analyzing six additional common SNPs, none of which was associated with otosclerosis. In aggregate, our data suggest that common variants in *BMP2* and *BMP4* are not a major genetic contributor to otosclerosis in this German population.

While common variation may not make a major contribution to otosclerosis pathogenesis, patient-specific rare variation may play a role. We identified 7 novel rare variants by sequencing *BMP2* and *BMP4* in all patients and controls. Four of these variants were within the coding region and unique to patients. One, a synonymous change, was not studied, but the other three variants (two in *BMP2* and one in *BMP4*) were analyzed for their ability to phosphorylate the receptor Smad for BMP2/4 signaling, Smad1/5/8. Although the *BMP2*^{R105Q} variant did not change the signaling capability of BMP2 as compared to wild type, the *BMP2*^{K357-R396del} variant was associated with decreased Smad1/5/8 phosphorylation in treated MG-63 cells. It is known that BMP2 null mice die between E7-E10.5 however the patient carrying this variant has no relevant detectable health issues other than otosclerosis.

The N150K variant in *BMP4* was also associated with decreased Smad1/5/8 phosphorylation in treated MG-63 cells. This variant has been reported in two children with anomalous kidney development: in the heterozygous state in an individual with renal hypodysplasia, and in the homozygous state in an individual with renal cystic dysplasia (40). Subsequent analysis of this variant by Tabatabaieifar and colleagues showed that *BMP4*^{K150} is retained in the perinuclear regions of the endoplasmic reticulum in COS7 cells (43). Although we did not analyze its subcellular location in HEK293T cells, our data suggest that enough *BMP4*^{K150} is produced and secreted from transfected HEK293T cells to affect the downstream modulator Smad1/5/8. To our knowledge our patient has not been diagnosed with any form of kidney disease, and neither of the patients described by Tabatabaieifar and colleagues is reported to have otosclerosis.

While *in silico* analysis predicted the *BMP4*^{K150} variant to be benign, our functional analyses showed that the variant affects downstream Smad signaling, a result that suggests caution when using *in silico* data to 'predict' pathogenicity. This study also highlights the importance of assessing functional relevance of newly identified genetic variants, as functional studies of different variants may highlight common changes in protein function. With personalized genomic medicine on the horizon, the relevance of these rare variants and their functional impact will be heightened. For example, while the current standard of care for otosclerosis is stapes surgery, further studies of the Smad signaling pathway may lead to other therapeutic options for select patients.

The loss of Smad1/5/8 signaling resulting from the BMP2^{K357-R396del} and the BMP4^{N150K} mutations suggests that loss of signaling contributes to the pathogenesis of otosclerosis. This study is the second to demonstrate abnormal function by osteogenic factors in otosclerosis. The first study demonstrated that a common SNP underrepresented in Belgian-Dutch and French otosclerosis patients caused an increase in TGF- β 1 signaling (44).

In aggregate, our results provide compelling evidence that reduced activity of TGF- β superfamily proteins may contribute to otosclerosis however more studies are needed to determine the effects of altered signaling by TGF- β superfamily members on bone remodeling. It is possible that reduced Smad signaling from variants in different components of the signaling pathway disturbs the static nature of the otic capsule and leads to active remodeling in the otic capsule in otosclerosis patients. Future studies looking at variants in all of the molecules in the Smad signaling pathway may show that a substantial percentage of the otosclerosis population carry variants that lead to altered Smad signaling.

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Figure 1. Rare coding variants found in *BMP2* and *BMP4*. Each gene encodes a protein that consists of a signal peptide, prodomain and a mature peptide. Amino acids for domain boundaries are indicated. Variants found in the German patient cohort are indicated by arrows.

Figure 2. Smad signaling affected by variants in BMP2 and BMP4. Conditioned media from transfected HEK293T cells show BMP2 or BMP4 expression. Phosphorylation levels of Smad1/5/8 were analyzed from MG-63 cells treated with conditioned media from transfected HEK293T cells.

Table 1
Common variation in *BMP2* and *BMP4* is not associated with otosclerosis in the German population.

SNP	Gene	Gender interaction	Additive ^a	Dom/Rec ^b	OR [95% CI]
rs2273073	<i>BMP2</i>	0.0719	0.8858	0.8858	0.919 [0.290–2.919]
rs1049007	<i>BMP2</i>	0.9369	0.5796	0.8626	0.979 [0.748–1.282]
rs235768	<i>BMP2</i>	0.5161	0.7603	0.6847	1.044 [0.797–1.367]
rs13037675	<i>BMP2</i>	0.0786	0.9040	0.8901	0.962 [0.521–1.775]
rs3178250 ^c	<i>BMP2</i>	0.3817	0.5628	0.6577	1.109 [0.792–1.552]
rs235769	<i>BMP2</i>	0.4412	0.8672	0.8608	0.975 [0.732–1.299]
rs17563 ^c	<i>BMP4</i>	0.1050	0.6985	0.9724	0.950 [0.739–1.223]
rs74054236	<i>BMP4</i>	0.0713	0.0670	0.0670	3.990 [0.801–19.873]

^a p-values for additive model of disease
^b p-values for dominant-recessive model of disease
^c SNPs previously associated with otosclerosis in Belgian-Dutch and French populations

Table 2

Variation found in the German population in the *BMP2* and *BMP4* genes.

	Total	% total variation	# Novel variants	% total novel variation	Novel variation	
					Cases	Controls
5'UTR	3	18.8	3	37.5	0	3
3'UTR	4	25.0	1	12.5	0	1
Synonymous	3	18.8	1	12.5	1	0
Missense	5	31.3	2	25.0	2	0
Nonsense	0	0.0	0	0.0	0	0
Splice site	0	0.0	0	0.0	0	0
Insertion/deletion	1	6.3	1	12.5	1	0
Total	16		8		4	4

Table 3

Power calculations for the German population for the *BMP2* and *BMP4* genes.

SNP	Previous reported data		Power Calculations	
	Gene	OR [95% CI] ^a	DAF ^a	German population
rs3178250	<i>BMP2</i>	2.027 [1.380–2.979]	0.247	54%
rs17563	<i>BMP4</i>	1.209 [1.070–1.370]	0.486	96%

^a Odds ratios and disease allele frequencies were obtained from data reported in the previous association study (24).