Mutations and altered expression of SERPINF1 in patients with familial otosclerosis

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Abstract

Otosclerosis is a relatively common heterogenous condition, characterized by abnormal bone remodelling in the otic capsule leading to fixation of the stapedial footplate and an associated conductive hearing loss. Although familial linkage and candidate gene association studies have been performed in recent years, little progress has been made in identifying disease-causing genes. Here, we used whole-exome sequencing in four families exhibiting dominantly inherited otosclerosis to identify 23 candidate variants (reduced to 9 after segregation analysis) for further investigation in a secondary cohort of 84 familial cases. Multiple mutations were found in the SERPINF1 (Serpin Peptidase Inhibitor, Clade F) gene which encodes PEDF (pigment epithelium-derived factor), a potent inhibitor of angiogenesis and known regulator of bone density. Six rare heterozygous SERPINF1 variants were found in seven patients in our familial otosclerosis cohort; three are missense mutations predicted to be deleterious to protein function. The other three variants are all located in the 5'-untranslated region (UTR) of an alternative spliced transcript SERPINF1-012. RNA-seq analysis demonstrated that this is the major SERPINF1 transcript in human stapes bone. Analysis of stapes from two patients with the 5'-UTR mutations showed that they had reduced expression of SERPINF1-012. All three 5'-UTR mutations are predicted to occur within transcription factor binding sites and reporter gene assays confirmed that they affect gene expression levels. Furthermore, RT-qPCR analysis of stapes bone cDNA showed that SERPINF1-012 expression is reduced in otosclerosis patients with and without SERPINF1 mutations, suggesting that it may be a common pathogenic pathway in the disease.

Introduction

Otosclerosis is one of the most common causes of hearing impairment among young adults. It is characterized by abnormal bone homeostasis within the otic capsule (1). The remodelling process often involves the stapedio-vestibular joint, and can lead to fixation of the stapedial footplate and a conductive hearing loss that result in clinical otosclerosis. Histological otosclerosis is seen more frequently than clinical otosclerosis; however in these cases, the stapes is not fixed and the diagnosis can only be made post-mortem (2). The age of onset is variable, although hearing loss in clinical otosclerosis typically begins in the third decade and progressively becomes more severe.

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Received: February 24, 2016. Revised: March 24, 2016. Accepted: March 25, 2016

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Hearing loss is bilateral in 70–85% of cases and is usually asymmetrical, developing initially in one ear before progressing to the other (3). Patients choose to undergo surgery to replace all or part of the affected stapes with a prosthetic device which ameliorates the hearing loss to varying degrees. Alternatively, the condition can be managed with hearing-aids (4).

The clinically relevant form of otosclerosis is relatively common in individuals of Indian and European extraction with a reported frequency of 0.3–0.4% in white Europeans; it is much rarer in African, Native American and other Asian populations (4–7). In most individuals, otosclerosis appears to be sporadic and is considered a complex condition with both environmental and genetic factors contributing (7–9). However, otosclerosis also occurs with a strong familial inheritance pattern in up to 50% of cases. The pattern of inheritance in familial otosclerosis is most often consistent with an autosomal dominant mutation that exhibits variable penetrance estimated at 80–90% (7).

To date, otosclerosis has proved resistant to analysis by conventional genetic techniques. Variable penetrance limits the power of linkage analysis, and so although eight genetic loci have been linked to otosclerosis these chromosomal regions are very large and contain many genes (10–17). As a result, no causal mutations underlying familial otosclerosis have yet been identified limiting progress in therapeutic development (7–9). With the recent advances in sequencing technologies, there have been numerous reports of novel genetic mutations identified using whole-exome sequencing (WES), including the identification of genes involved in rare Mendelian disorders that had previously eluded researchers (18). Furthermore, although more challenging, there is great interest in extending these technologies to common, complex traits (19). Otosclerosis is a heterogeneous disorder in the population but one that often occurs in multiple members of the same family consistent with a monogenic type of inheritance. Hence, a WES approach in these families may provide a powerful discovery tool to reveal the genetic causes and molecular pathways disrupted in otosclerosis. However, because of the frequency of the disease the causal variants are unlikely to be identified by WES alone as frequency filters will have to be set higher than in rare disorders. In this work, we used a combination of WES, RNA-seq and functional analysis to identify disease-causing genes in familial otosclerosis (Fig. 1A). In one family, we detected a rare heterozygous missense variant, c.601G > A, in SERPINF1 (Serpin Peptidase Inhibitor, Clade F; Fig. 1B). Five additional rare variants were identified in SERPINF1 from a cohort of 84 familial cases of otosclerosis. We analysed the effect of these mutations and identified SERPINF1 as the first disease-causing gene in otosclerosis.

**Results**

**WES in four families identifies candidate variants for otosclerosis**

We sequenced the exomes of 10 individuals from four families with familial otosclerosis, three of European ancestry and one of mixed European and Caribbean ancestry in which the otosclerosis has been inherited via the European ancestral line. Exome capture was performed with an Agilent SureSelect Human All Exon 50 Mb Kit and subject to massively parallel sequencing. An average of 16.5 Gb of sequence was generated per individual as paired-end 100 bp reads. Reads were mapped to the reference sequence (GRCh37_hs37d5) with 96.16% of the bases mapping at >10x coverage. The mean depth of coverage was 178.5-fold with an average of 89 656 variants

![Figure 1. Familial otosclerosis and the next-generation sequencing approach for discovering causal variants. (A) Schematic presenting the workflow implementing a combination of whole-exome sequencing, RNA-seq and functional analysis to identify disease-causing variants in familial otosclerosis. (B) Pedigree of Family 8 shows an autosomal dominant inheritance pattern, consistent with familial otosclerosis. A rare heterozygous missense variant, c.601G > A, was identified in SERPINF1. Open symbol, clinically unaffected; black symbol, confirmed otosclerosis; light grey symbol, other hearing loss; arrow, proband.](http://hmg.oxfordjournals.org/)

at University College London on January 13, 2017
identified per individual. A filtering strategy was applied to the data in order to prioritize the identified variants (Table 1).

Firstly, variants were filtered within families so that only variants common to all affected individuals were retained. We focused on non-synonymous variants, those affecting splice sites and insertions–deletions (indels) in coding genes. Variants in non-coding genes (with the exception of micro RNAs) and changes within non-coding regions of genes that were not predicted to affect splicing were filtered out. Known variants with a frequency >0.02 were filtered out based on the September 2011 release of 1000 Genomes Project and 500 Exomes Project. This was based on a reported otosclerosis frequency of 0.003–0.004 in Europeans (4,6), of which up to 50% are familial with a dominant inheritance meaning that a familial otosclerosis causal variant would have a frequency of 0.0015–0.002 if homogenous. A filter set 10-fold higher than this is assumed to retain the causal variant especially as there is good evidence that familial otosclerosis is heterogeneous. At this stage, two parallel filtering pipelines were applied (Table 1). These were (i) a robust ‘naive pipeline’ based on retaining variants predicted to be deleterious by either PolyPhen-2 (20) or SIFT (21) and absent from dbSNP134; and (ii) a ‘candidate-gene pipeline’ where only variants in an otosclerosis candidate gene list of 494 genes were retained. The list included genes implicated in otosclerosis from association studies (22–26), gene expression studies (27), genes within linked regions of the genome (10–17,28) and genes known to be involved in other connective tissue disorders that can exhibit an otosclerosis-like hearing loss (29–33). The list also included 176 genes found to be differentially expressed in otosclerotic stapes by an RNA-seq analysis of 12 stapes (J.L. Ziff, J.A. Lavy, S.R. Saeed and S.J. Dawson, manuscript in preparation). Finally, for both pipelines variants were removed if they were also present in a previous in-house exome sequencing project of 20 individuals without otosclerosis.

The remaining variants from both pipelines were annotated and 23 prioritized for follow-up firstly in segregation analysis (Supplementary Material, Table S3). Prioritization was based on a number of factors including GERP score, predicted effect of the variant, expression in otosclerotic and control stapes and known biological role. Fourteen of 23 variants were ruled out due to lack of familial segregation leaving nine candidate variants in the following genes: ANKS1A (c.1291C > A), SERPINF1 (c.601G > A), VPS53 (c.107C > G), TRIM17 (c.915C > G), COL1A2 (c.808G > A), Fzd2 (c.655C > T), GNGT1 (c.82C > A), mir183 (N.81G > T) and ZNF225 (c.1698A > T).

Table 1. Variant filtering process applied to whole-exome sequencing data in four families with otosclerosis

| Filters | Number of retained variants following application of filter |
|---------|----------------------------------------------------------|
|         | Family A | Family B | Family C | Family D | Average |
| All variants | 88 295 | 93 972 | 88 373 | 87 983 | 89 656 |
| Quality score > 30 | 82 689 | 87 135 | 82 983 | 83 058 | 83 966 |
| Present in all affected individuals in family | 58 538 | 65 275 | 58 775 | 55 454 | 59 511 |
| Exclude variants >0.02 freq 1000G | 5924 | 8339 | 5844 | 5865 | 6493 |
| Exclude variants in 500 Exome Project\* | 4001 | — | 3910 | 3925 | 5044 |
| Exclude synonymous variants | 729 | 1540 | 695 | 757 | 950 |
| Exclude variants in non-coding genes | 278 | 873 | 279 | 285 | 429 |
| Exclude variants in unaffected individuals\* | — | 314 | — | — | 289 |

Candidate filters

| Filters | Cand | Naive | Cand | Naive | Cand | Naive | Cand | Naive | Naive |
|---------|------|-------|------|-------|------|-------|------|-------|-------|
| Present in candidate gene list | 26 | 112 | 26 | 158 | 27 | 94 | 29 | 112 |
| Exclude homozygous alleles\* | 18 | 28 | — | — | 17 | 29 | — | — |
| Exclude if on in-house WES database | 8 | 22 | 12 | 44 | 3 | 21 | 8 | 27 |
| Combined | 30 | 56 | 23 | 35 |
| Prioritized for follow up | 4 | 8 | 4 | 7 |

| Two affected individuals were subject to exome sequencing in families A, B and C and three in Family D. One unaffected individual was also subject to exome sequencing in Family B. After initial filtering (upper panel), variants were filtered in two parallel strategies (lower panel); a candidate gene filter and a robust naive filter based on predicting deleterious variants and rarity. The retained variants were then combined and prioritized for follow up.
| Not applied to Family B as the ethnicity of this cohort was not applicable.
| Only applied to Family B as only family with unaffected individual.
| Not applied to Family B or Family D as both originated from relatively small communities, highlighting a small possibility of the condition exhibiting an autosomal recessive inheritance pattern. |
otosclerosis by Sanger sequencing of the exon and exon/intron boundaries. None of the variants listed above was detected in the cohort of 53 unrelated cases of familial otosclerosis. However, a second rare non-synonymous variant (c.441G > C) was found in the SERPINF1 gene in two unrelated individuals. Exon 5 of SERPINF1 was subsequently sequenced in a further 31 unrelated individuals with a family history of otosclerosis identifying a novel 3 bp deletion (c.440-38delTCG) in one individual. None of these variants was found in 175 control samples sequenced. Further sequencing of all coding exons and intron–exon junctions in SERPINF1 in the otosclerosis cohort revealed three additional rare non-synonymous mutations in exon 3 and exon 4 of SERPINF1 (c.167C > G, c.331G > A, c.392C > A). In summary, six rare heterozygous variants were identified in seven unrelated patients in the SERPINF1 gene (see Table 2). Allele frequencies of the SERPINF1 variants identified were much greater in the unrelated otosclerosis cohort compared with their reported frequencies in the variant databases, 1000 Genomes and NHLBI Exome Sequencing Project.

Table 3. Summary of in silico and functional analysis of identified SERPINF1 variants

| Transcript SERPINF1-001 | Transcript SERPINF1-012 |
|-------------------------|-------------------------|
| Nucleotide exchange | Exon | Protein effect | PolyPhen-2 (HumDiv Score) | MutationTaster (probability) | Nucleotide exchange | Exon | Mfold | TF binding | Luciferase activity |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| c.167C > G | 3 | p.Ala56Gly | Probably damaging (0.988) | Disease causing (0.999) | — | — | — | — | — |
| c.331G > A | 4 | p.Asp111Asn | Possibly damaging (0.879) | Disease causing (0.999) | — | — | — | — | — |
| c.392C > A | 4 | p.Ala131Asp | Probably damaging (0.969) | Disease causing (0.707) | — | — | — | — | — |
| c.440-38delTCG | — | — | — | Polymorphism (0.999) | c.-202_200 | 1 | S’-UTR | Affected | Affected |
| c.441G > C | 5 | p.Lys147Asn | Benign (0.135) | Disease causing (0.999) | c.-161G > C | 1 | S’-UTR | No change | Affected |
| c.601G > A | 5 | p.Asp201Asn | Benign (0.000) | Polymorphism (0.999) | c.75G > A | 1 | S’-UTR | No change | Affected |

aGenBank: NG_028180.1; Ensembl Transcript: ENST00000254722.
bEnsembl Transcript: ENST00000573763.
suggestions that the SERPINF1-012 transcript, which contains exons 5–8 only, is the major SERPINF1 isofrom in the stapes. Additionally, analysis of stapes tissue from the proband in Family B with the c.601G>A variant in the 5′-UTR of SERPINF1-012 indicated that levels of this transcript are reduced in this individual suggesting that they do affect expression. Moreover, the average read counts for SERPINF1 exons 5–8 were significantly reduced in otosclerotic stapes (n = 8) compared with control stapes (P < 0.05; Fig. 2B), whereas no significant differences were observed for the average read counts in exons 1–4 between control and otosclerotic stapes.

Expression of SERPINF1 transcripts was further investigated in a second, larger sample of stapes using RT-qPCR. This constituted stapes suprastructure from 75 unrelated affected individuals, 5 control individuals and 1 affected individual with the c.441G>C mutation in the 5′-UTR of SERPINF1-012. RT-qPCR was used to determine the relative expression of upstream (assay spanning exons 3 and 4) and downstream exons (assay spanning exons 6 and 7), which relate to SERPINF1-001 and SERPINF1-012 transcripts, respectively (see schematic in Fig. 2A). A large 2.4-fold reduction was found for the downstream assay in the stapes from the individual with the c.441G>C mutation compared with controls suggesting that this SERPINF1-012 transcript is affected by the variant in the 5′-UTR of this transcript (P < 0.0005; Fig. 2D). In addition, we found that the level of SERPINF1 mRNA is significantly reduced in the general otosclerosis sample for both assays compared with controls (Fig. 2C and D). This reduction is greater for the downstream exons (1.9-fold, P < 0.00005) contained within SERPINF1-012 than for upstream exons (1.3-fold, P < 0.05), consistent with RNA-seq data.

Investigating the effect of SERPINF1-012 5′-UTR variants on expression

Annotation of non-coding variants and prediction of their effects are still limited, meaning that the evaluation of effect of the 5′-UTR variants on SERPINF1 requires further investigation. Data from RT-qPCR and RNA-seq indicated that two of the mutations in the 5′-UTR reduce SERPINF1-012 expression in stapes removed from these individuals. 5′-UTR variants can affect regulation of both transcription and translation, either by affecting the binding of transcription factors or by altering the stability of the mRNA through changes in secondary structure. In silico analysis using a transcription factor prediction tool, MathInspector (35), predicted that the three SERPINF1-012 5′-UTR variants are within various transcription factor binding sites (see Supplementary Material, Table S4). Most notably, both the c.440-40_440-38delTCG and c.601G>A variants were predicted to create binding sites for transcription factors involved in bone regulation; PAX3 (c.440-40_440-38delTCG), RBPJ and NFYA (c.601G>A) (36–38). We also investigated the effects of the 5′-UTR variants on RNA folding using Mfold (39), which predicts RNA structure based on free energy minimization. RNA structures of the 5′-UTR alone and the full length SERPINF1-012 mRNA were predicted (Supplementary Material, Fig. S1). The c.440-40_440-440-
38delTCG variant showed a marked difference in both the 5'-UTR and full mRNA folding predictions.

To confirm whether the 5'-UTR mutations have effects on protein production, luciferase constructs containing wild-type and mutant 5'-UTR sequences were tested in MG-63 cells (Fig. 3A). Compared with the wild-type construct, the c.440-40_440-38delTCG and c.441G>C alleles showed a significantly reduced level of luciferase activity (Fig. 3B), indicating that these variants will likely reduce the translational efficiency of the SERPINF1-012 transcript. The c.601G>A allele also had a significant influence on luciferase activity; however, a significant increase in translational activity was observed.

**Discussion**

Despite its high prevalence and disease burden, the impact of hearing impairment on quality of life is often underestimated. Hearing loss can lead to social isolation and depression, and is associated with cognitive decline (40–44). Otosclerosis is one of the most common causes of hearing impairment in young adults and has a significant detrimental effect upon patients (45). Despite the importance of the disease, the genetic pathways and aetiology of otosclerosis remain poorly understood. Using a WES approach we identified a rare SERPINF1 mutation in a family affected by an autosomal dominant otosclerosis. Five additional mutations were identified in six individuals across a cohort containing 84 unrelated individuals with a family history of otosclerosis meaning that 8.0% of familial otosclerosis across a cohort containing 84 unrelated individuals with a familial history of otosclerosis is predicted to have benign effects on

**Figure 3.** Mutations in the 5'-UTR influence translational efficiency of the SERPINF1-012 transcript. (A) Fragments containing the wild-type or mutant 5'-UTRs of the SERPINF1-012 transcript were cloned into the pGL4.10 expression vector and transfected into MG-63 cells. (B) For the luciferase assay wild-type values were set to 1, to which mutant values were normalized. These results are the average of 12 repeats in four independent experiments. The c.440-40_440-38delTCG (c.38pdel) and c.441G>C mutations show a significant reduction in translational activity, whereas the c.601G>A mutation displays a significant increase. Error bars indicate standard error of mean (*P < 0.05; **P < 0.01 in a Student’s two-tailed t-test versus control data).
otosclerosis has not been defined. The identification of SERPINF1 mutations in patients with familial otosclerosis in this study and the altered stapes expression observed in the gene that encodes PEDF provide a new link between heparan sulphate and modulation of TGFβ signalling in otosclerosis. Furthermore, heparan sulphate has been shown to compete with PEDF in interactions with collagen, due to overlapping binding sites (55), highlighting a possible functional relationship between collagen, PEDF and heparan sulphate during angiogenesis. Some of the residues involved in heparan binding are absent from the shorter alternatively spliced SERPINF1-012 transcript, which could have a significant impact on its role in angiogenesis and bone regulation in the stapes compared with SERPINF1-001 in other bone.

Mutations in SERPINF1 are already known to underlie one recessive form of the connective tissue disorder osteogenesis imperfecta (OI) type VI (MIM: 613982). OI is a brittle bone disorder caused by mutations in COL1A1 or COL1A2 in over 90% of cases; with mutations in many other genes, including SERPINF1, responsible for the other 10% of cases. To date 20 unique sequence variants in SERPINF1 have been identified in OI type VI patients (56–65). Most of the reported variants are nonsense or frameshift mutations that are expected to cause mRNA instability due to nonsense-mediated decay, leading to complete loss of PEDF expression (60). The three SERPINF1 in-frame deletions or insertions identified in OI lead to retention or degradation within cellular compartments and thereby interfere with PEDF secretion (65). Although it is expected that a loss of PEDF results in production of undermineralized bone, the mechanism by which this occurs is unknown. In bone, as PEDF binds to collagen with high affinity and is actively expressed in osteoblastic regions of active bone formation, it is possible that PEDF plays an important functional role in bone matrix remodelling (67). Some patients with OI experience conductive hearing loss similar to that of otosclerosis. These similarities have previously led researchers to postulate that otosclerosis and OI may have a common aetiology (68). Therefore, given SERPINF1’s known role in bone regulation these data suggest SERPINF1 is an excellent candidate for an otosclerosis gene. Importantly, all of the otosclerosis patients with SERPINF1 mutations display no OI and have not reported a disproportionate amount of broken bones. One patient reported having osteoporosis and rheumatoid arthritis.

In contrast to OI, none of the mutations described here in the otosclerosis cohort are nonsense or frameshift mutations, so it is unlikely that they would lead to nonsense mediated decay. The variants identified were found to have a more subtle effect on SERPINF1 function. The three missense variants predicted to be deleterious to the protein function of SERPINF1 (c.167C>G, c.331G>A and c.392C>A) would be expected to only cause a partial loss of PEDF function rather than the complete loss of PEDF expression seen in OI patients. The c.440-40,440-38delTCG, c.441G>C and c.601G>A variants identified in the 5′-UTR of SERPINF1-012 were shown to have a significant impact on the translational efficiency of this transcript; therefore, it is possible that these variants primarily affect an alternatively spliced transcript which is critical for stapes maintenance but redundant in other bone (Fig. 4). This could explain the phenotypic differences seen between OI and otosclerosis and would support the suggestion that they have a common aetiology.

The results from this study indicate that WES is a useful tool for investigating disease-causing variants in families exhibiting autosomal dominant inheritance of otosclerosis. The step-wise variant prioritization process has been effective at narrowing down the large number of potential disease-causing variants from an average of 89,656 variants per family to a small pool of those that are most likely to be involved in the disease process. Ultimately, our results demonstrate the power of an exome sequencing approach over linkage analysis in familial cases of a relatively common disorder, even in the presence of variable penetrance and heterogeneity. It also shows the value of transcriptome data in the filtering and prioritization of variants, highlighting SERPINF1 as the first disease-associated gene to be identified in otosclerosis.
Materials and methods

Patient recruitment

Individuals with a confirmed diagnosis of otosclerosis were recruited from the Royal National Throat Nose and Ear Hospital, London, UK and The Princess Margaret Hospital in Windsor. The study was approved by the London Bloomsbury NRES Ethics committee (11/LO/0489) and patients were recruited by informed consent. From this cohort, a sub-cohort of individuals with evidence of familial otosclerosis was identified based on patient questionnaire data of family history (defined as two or more relatives with otosclerosis). Blood or saliva samples (Oragene® Saliva Kit, DNA Genotek) were obtained for genomic DNA isolation by standard methods. For those patients undergoing stapedotomy surgery to remove the stapes superstructure, the tissue was retained for gene expression studies. Control samples were obtained from individuals where the stapes was removed as part of surgery for a number of other indicators including head trauma, glomus tumour and during total petrosectomy.

WES and filtering

WES was performed at the Wellcome Trust Sanger Institute on genomic DNA isolated from each of the 10 individuals selected from four families with familial otosclerosis. The sequencing data have been deposited at the European Genome-phenome Archive (EGA; EGAS00001000156). After target enrichment using Agilent SureSelect Human All Exon V3 kit the whole-exome DNA library from each participant was sequenced using Illumina HiSeq 2000 with 100 bp paired-end reads and mapped to the reference human genome (GRCh37_hg19) based on the sequence alignment. Both single-nucleotide variants and indels were identified using SAMtools (69), GATK (70) and Dindel (71). A mean of 16.5 Gb of sequence was generated per sample with an average of 96.16% of the bases mapping to the reference genome at a coverage of at least 10×. The mean depth of coverage was 178.5×. Of all the variants mapped, 93.7% met a quality score threshold of 30. The filtering strategy applied to the data is described in Table 1 and in the main text.

Segregation analysis and mutation detection

All variants prioritized for follow-up were confirmed by Sanger sequencing in the relevant family. Genotyping for segregation analysis was performed by Sanger sequencing. In segregation analysis, variants were excluded if they were present in more than one unaffected individual above the age of onset of otosclerosis in that family. Variants were also excluded if they were not present in all individuals with otosclerosis. Sanger sequencing was used for genotyping in the unrelated familial otosclerosis cohort. PCR primers used to amplify the SERPINF1 exons are shown in Supplementary Material, Table S1.

RNA-seq and RT-qPCR analysis

Human stapes were preserved in AllProtect™ Solution (Qiagen) and stored at −80°C. Stapes were homogenized in QIAzol Lysis reagent and RNA purified using the RNeasy lipid tissue mini kit (Qiagen) according to the manufacturer’s protocol, including an on column DNase digest. RNA was reverse transcribed into cDNA by Clontech SMARTer™ kit for RNA-seq and by Omniscript Reverse Transcriptase Kit for RT-qPCR. The integrity of the cDNA for RNA-seq was confirmed on an Agilent Bioanalyzer 2100 before sending to Orogenetics Corp (Norcross, GA, USA) where cDNA fragmentation was performed on the samples and cDNA libraries constructed before 100 bp paired-end RNA sequencing using Illumina HiSeq 2000 was carried out. Paired-end 100 nt reads were aligned to human genomic assembly hg19 and visualized on the DNAanexus platform (Mountain View, CA, USA). Taqman® gene expression assays were performed on human otosclerotic and control cDNA samples. RT-qPCR was conducted using an SDS7500 real time PCR machine (Applied Biosystems) with primer/probe pairs obtained from Applied Biosystems. SERPINF1 assays spanning exons 3–4 (Assay ID: Hs011006934_m1) and exons 6–7 (Assay ID: Hs011006937_m1) were performed with eukaryotic 18s RNA as an endogenous control. Relative quantification of SERPINF1 was calculated relative to a calibrator sample (control stapes) using the ΔΔCt method.

In silico analysis of 5′-UTR variants

MatInspector (35), a transcription factor prediction tool, was used to identify potential transcription factor binding sites in the region where the variants in the 5′-UTR of the SERPINF1-012 transcript were found. The matrix score calculates a match between the sequence and the matrix, ranging from 0 to 1, with 1 indicating an exact match. Putative RNA folding structures were predicted using Mfold (39) with standard settings.

Cloning of SERPINF1-012 5′-UTR variants

The Gibson assembly cloning method (72) was used to insert the DNA fragments of three SERPINF1-012 5′-UTR variants into pGL4.10 vector separately, as well as a wild-type control. Gibson assembly primers for SERPINF1-012 fragments were synthesized (Supplementary Material, Table S2), and inserts were prepared by PCR amplification using the Q5 High-Fidelity PCR kit (New England Biolabs) and gDNA as a template. PCR products were used in DNA assembly reaction with PCR-linearized pGL4.10 vector using Gibson Assembly Cloning kit (New England Biolabs). Each assembly reaction contained approximately 150ng of insert and 50ng of the expression vector and incubated at 50°C for 1h following the manufacturer’s protocol. After the assembly reaction, the reaction mix was transferred into NEB 5-alpha competent E. coli strain (New England Biolabs). After an overnight growth at 37°C, the pGL4.10 plasmids containing respective inserts were extracted using a QIaprep Spin Miniprep Kit (Qiagen) and constructs were verified by Sanger sequencing.

Transfection and luciferase activity assay

To measure the translational activity of the 5′-UTR variants of SERPINF1-012, dual-luciferase reporter assays were conducted in MG-63 cells. The human osteosarcoma MG-63 cell line was obtained from Dr Vehid Salih at the Eastman Dental Hospital, University College London. MG-63 cells were cultured in Dulbecco’s modified Eagle medium (DMEM; Invitrogen) and were maintained at 37°C in a 5% CO2 incubator. Medium was supplemented with glutamax, 10% foetal bovine serum and 1% penicillin/streptomycin. MG-63 cells (1 × 10⁴) were seeded on 6-well plates and transfected 24h later using the calcium phosphate method (73). A total of 270 ng plasmid DNA (250 ng of pGL4.10 constructs and 20 ng phRL-null) was used for each
transfection. After incubation for 16 h, cells were exposed to glycerol shock condition with 2 ml of 15% glycerol containing DMEM for 2 min 30 s. Subsequently, the mixture was aspirated and replaced with fresh DMEM supplemented with glutamax, 10% foetal bovine serum and 1% penicillin/streptomycin. Cells were harvested 48 h after transfection, and luciferase activities were measured with the Dual-Luciferases® Reporter Assay System (Promega) and a single-tube luminometer (Turner Biosystems). Luciferase activities shown in figures are the mean of 12 transfections in four independent experiments.

Web resources
The URLs for data presented herein were last accessed on February 23, 2016 and are as follows:
1000 Genomes, http://www.1000genomes.org/
Ensembl, http://www.ensembl.org/
European Genome-phenome Archive (EGA), https://www.ebi.ac.uk/ega/
MatInspector, https://www.genomatix.de/matinspector
Mfold, http://mfold.rna.albany.edu/?q=
MutationTaster, http://sift.jcvi.org/
NHLBI ESP Exome Variant Server, http://evs.gs.washington.edu/EVS/
OMIM, http://www.omim.org/
PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/
SIFT, http://sift.jcvi.org/

Supplementary material
Supplementary Material is available at HMG online.

Acknowledgements
We are indebted to the patients and their families for their willingness to participate in our research study. We are also grateful for the physicians and hospital staff, whose efforts in collecting these samples were essential to this research.

Conflict of Interest statement. None declared.

Funding
This work was supported by Deafness Research UK (512UEI:SD, 563:UEI:SD); Action on Hearing Loss (G71); the British Society of Audiology (BSA Applied Research Fund). Exome sequencing was performed by the Wellcome Trust Sanger Institute supported by the Wellcome Trust Sanger Institute supported by a Wellcome Trust grant (098051). Funding to pay the Open Access publication charges for this article was provided by the Wellcome Trust.

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