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Published in:
Human Genetics

DOI (link to publication from Publisher):
10.1007/s00439-021-02334-8

Publication date:
2022

Document Version
Accepted author manuscript, peer reviewed version

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ABSTRACT

In this study we investigated the association of *ACAN* variants with otosclerosis, a frequent cause of hearing loss among young adults.

We sequenced the coding, 5'-UTR and 3'-UTR regions of *ACAN* in 1497 unrelated otosclerosis cases and 1437 matched controls from six different subpopulations. The association between variants in *ACAN* and the disease risk was tested through single variant and gene-based association tests.

After correction for multiple testing, 14 variants were significantly associated with otosclerosis, ten of which represented independent association signals. Eight variants showed a consistent association across all subpopulations. Allelic odds ratios of the variants identified four predisposing and ten protective variants. Gene-based tests showed association of very rare variants in the 3'-UTR with the phenotype.

The associated exonic variants are all located in the CS domain of *ACAN* and include both protective and predisposing variants with a broad spectrum of effect sizes and population frequencies. This includes variants with a strong effect size and low frequency, typical for monogenic diseases, to low effect size variants with high frequency, characteristic for common complex traits. This single-gene allelic spectrum with both protective and predisposing alleles is unique in the field of complex

diseases. In conclusion, these findings are a significant advancement to the understanding of the etiology of otosclerosis.

Key words: Otosclerosis, Aggrecan, Hearing loss, Genetic association, Complex genetics

DECLARATIONS

Funding

This study was supported by funding from the Belgian Science Policy Office Interuniversity Attraction Poles (BELSPO-IAP) program (project IAP P7/43-BeMGI to G.V.C.) and the Obelske Familiefond (to A.T.H. and M.B.P.).

Conflits of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Availability of data and material

The datasets generated and/or analyzed during the current study are available in the European Genome-phenome Archive (EGA; EGAS00001004398).

Code availability

Not applicable

Authors' contributions

G.V.C. and E.F. conceived and designed the study and were in charge of overall direction and planning. V.T., I.S, I.D, A.H, D.Z and H.P.M.K. recruited patients and controls in the participating centers and collected samples. A.T.H., M.S. and A.H. designed the smMIPs experiment. A.T.H., L.J.M.T and M.S performed the laboratory experiments and collected data in the lab. E.F. performed the statistical analyses. A.T.H., L.J.M.T, I.S., G.V.C. and E.F. all contributed to the interpretation of the results. A.T.H.

and L.J.M.T. have drafted the manuscript and I.S., M.B.P, G.V.C and E.F. substantially revised it. All authors critically read and approved the final manuscript.

Ethics approval and consent to participate

Informed consent was obtained from all participants and local ethics committees approved all procedures in accordance with the World Medical Association's Declaration of Helsinki.

Consent for publication

Not applicable

INTRODUCTION

Otosclerosis (MIM 166800) is a multifactorial disorder characterized by abnormal bone remodeling in the human middle and inner ear (Milroy and Michaels 1990). Discrete areas of endochondral bone in the otic capsule are replaced by otosclerotic bone. The disorder can lead to conductive hearing loss mostly due to the fixation of the stapes footplate (Quesnel et al. 2018). Also, in about 10% of affected persons, a sensorineural component is present caused by the invasion of otosclerotic foci into the cochlear endosteum. Otosclerosis usually debuts in the third or fourth decade of life and a prevalence of 0.3% to 0.4% has been reported in the white population (Declau et al. 2001).

In 50-60% of otosclerosis patients a positive family history is reported (Gordon 1989), where it presents with an autosomal dominant inheritance pattern with reduced penetrance (Moumoulidis et al. 2007). Large families with multiple otosclerosis patients have been analyzed with linkage analysis. So far, eight different loci (*OTSC1-5*, *OTSC7-8* and *OTSC10*) have been identified in eight different families (Bel Hadj Ali et al. 2008; Brownstein et al. 2006; Chen et al. 2002; Schrauwen et al. 2011; Thys et al. 2007b; Tomek et al. 1998; Van Den Bogaert et al. 2004; Van Den Bogaert et al. 2001). Despite

this success, the causative genes in these loci have not yet been identified. Potential candidate genes, including *NOG*, *COL1A1* and *COL1A2,* have been sequenced, but no causative variants were identified (Brown et al. 2002; Sykes et al. 1986; Sykes et al. 1990).

In cases where no positive family history is present, otosclerosis behaves as a complex disease caused by both genetic and environmental factors. Various genetic association studies for complex otosclerosis have been performed with varying results. The strongest associations have been found in *TGFB1*, *BMP2*, *BMP4*, *COL1A1* and *TNFRSF11B* and possibly other genes plays a role in the pathogenesis of otosclerosis (Chen et al. 2007; Khalfallah et al. 2011; McKenna et al. 1998; Priyadarshi et al. 2015; Schrauwen et al. 2012; Schrauwen et al. 2008; Sommen et al. 2014; Thys et al. 2007a). To date, two genome wide association studies (GWAS) have been performed, showing strong association signals in *RELN,* 11q13.1, *TGFB1, MEPE* and genes involved in bone remodeling (Rämö et al. 2020; Schrauwen et al. 2009). Next-generation sequencing (NGS) in otosclerosis research has led to succesful identification of pathogenic variants in *MEPE* (Schrauwen et al. 2019) and *SERPINF1* (Ziff et al. 2016), although the pathogenic role of the latter is under debate (Valgaeren et al. 2019).

In this study, we investigated the role of the *ACAN* gene in otosclerosis. The *ACAN* gene codes for the aggrecan protein, which is essential for cartilage function and skeletal development (Aspberg 2012). The protein consists of three globular domains (G1, G2 and G3), an interglobular domain between G1 and G2, a keratan attachment domain and a large chondroitin sulfate attachment (CS) domain (Kiani et al. 2002).. Aggrecan plays an important role in the extracellular matrix (ECM) of cartilage, where it interacts with different cell surface receptors, storage and release of signaling factors, cell division and migration and regulates the functional stability of the matrix (Aspberg 2012). Within the ECM, different proteoglycans play a role during skeletal developmaent (Ovadia et al. 1980). In particularly, aggrecan is important for the regulation of growth factors and signaling molecules during development of cartilage and plays a role in chondrocyte orginization, morphology and survival during embryonic limb development (Lauing et al. 2014). During endochondral ossification, the expression of

aggrecan dominates over other proteoglycans (Ovadia et al. 1980). Besides its role in the ECM, aggrecan has important functions in the growth plate. Numerous sulfated glycosaminoglycans (GAG) attach to the core protein of aggrecan and create a large, highly negatively-charged molecule. This allows hydration of the cartilage tissue and binding of growth factors and morphogens crucial to chondrocyte maturation and function (Kiani et al. 2002; Ruoslahti and Yamaguchi 1991). Predisposing variants in *ACAN* have been reported to cause advanced bone age, early-onset osteoarthritis, osteochondritis dissecans and short stature (Nilsson et al. 2014; Stattin et al. 2010). *ACAN* has been suggested to play a role in hearing disorders. Mice carrying a mutant aggrecan show hearing impairment (Yoo et al. 1991). For otosclerosis, it became a gene of interest when the OTSC1 locus was identified at 15q25-q26 (Tomek et al. 1998). A locus for autosomal dominant hearing loss, at chromosomal location 15q25-26, was found in proximity of the *ACAN* gene, overlapping with the OTSC1 locus (Mangino et al. 2001). A genome-wide association study into age-related hearing impairment (ARHI) showed a genome-wide significant association signal in variants close to the *ACAN* gene (Hoffmann et al. 2016b). A link between otosclerosis and ARHI has previously been hypothesized, through age-related bone mass loss in the cochlear capsule (Clark et al. 1995). More recently, in a talk by Dawson et al. (Dawson et al. 2018) at the Molecular Biology of Hearing and Deafness meeting in 2018 a high number of rare variants in *ACAN* was reported on the basis of whole exome sequencing of familial otosclerosis patients. In combination, these studies suggest a role for *ACAN* in different hearing disorders, including otosclerosis. In this study, we used a large set of unrelated cases, including both sporadic and familial cases, and controls to test if variants in the *ACAN* gene are involved in the occurrence of otosclerosis.

MATERIAL AND METHODS

Study population

All patients and controls were recruited at Center of Medical Genetics, University Hospital of Antwerp (Edegem, Belgium), Department of Clinical Sciences and Community Health, University of Milan

(Milan, Italy), Jean Causse Ear Clinic (Colombiers, France), ENT Department, Iuliu Hatieganu University of Medicine and Pharmacy (Cluj-Napoca, Romania), Department of Otolaryngology, University Hospital of Antwerp (Antwerp, Belgium), GZA Hospital campus Sint-August (Antwerp, Belgium), University Hospital of Ghent (Ghent, Belgium), Radboud University Medical Center (Nijmegen, the Netherlands) and University Hospital Zurich (Zurich, Switzerland).

Across all participating centers, otosclerosis diagnosis was based on surgical findings during stapes microsurgery or a combination of audiological and clinical data. Audiological and clinical data include medical history, otoscopy, tympanometry, acoustic reflex testing and audiometry. Both pure-tone audiometry (at 125, 250, 500, 1000, 2000, 4000, and 8000 Hz) and bone conduction (at 250, 500, 1000, 2000 and 4000 Hz) were measured. Tympanometry and stapedial reflexes were used to determine the fixation or mobility of the stapes.

The case group consisted of sporadic and familial cases. All cases were unrelated and one member of each family was selected. Controls were randomly collected by the same recruiting centers that collected the cases, matching for age, gender and ethnicity. Since the prevalence of otosclerosis in the general population is low, controls were not screened for absence of otosclerosis.

The study was approved by the Ethical Committee of the University of Antwerp (UA A10-07). Informed consent was obtained from all participants and local ethics committees approved all procedures in accordance with the World Medical Association's Declaration of Helsinki. Genomic DNA was isolated from either frozen or fresh blood samples using standard techniques.

Target enrichment, sequencing and quality control

234 single molecule molecular inversion probes (smMIPs) covering the coding region and 5'- and 3'-untranslated regions (UTR) with at least 10 bases overhang of the exon-intron boundaries of the *ACAN* gene were designed using MIPgen (Boyle et al. 2014) and ordered from Integrated DNA Technologies (Coralville, Iowa, USA). smMIP-enrichment was performed in accordance with protocols described

elsewhere with minor modifications (Supplementary Material and Methods)(Hiatt et al. 2013; O'Roak et al. 2012; Schrauwen et al. 2019). Sequencing was carried out on the NextSeq 500 (Illumina, San Diego, California, USA). VCF-files were generated using an in-house bioinformatics pipeline. Briefly, fastq reads were aligned to the human genome (version hg19) using BWA (v0.7.4). Afterwards, overlapping fragments of each read-pair were trimmed and PCR duplicates were removed based on the 8 bp random nucleotide tag. Unified Genotyper from GATK (v3.5.0) was used for multi-sample variant calling. Variants were annotated using ANNOVAR, which includes annotation to dbNSFP and dbscSNV. To reduce false positive variants, only samples with >10x coverage of at least 80% of the target region were retained for the analyses. Validation of resulting variants was carried out by comparison with frequent variants in GnomAD v2 (Karczewski et al. 2019) and by Sanger sequencing. Quality parameters for validation by Sanger sequencing were based on previous studies (Schrauwen et al. 2019; Valgaeren et al. 2019) (Supplementary Material and Methods).

Statistical analyses

For statistical analyses, the case group consisted of unrelated individuals and no more than one member of each family. Statistical tests into the association between the *ACAN* genotypes and the disease status were carried out using the vtools software package (San Lucas et al. 2012). Single variant association tests were performed for all variants using the Fisher's Exact Test. Genomic inflation factors were calculated based upon results from previous association studies on the same case-control panel. This includes the study on a previously tested candidate gene *SERPINF1* (Valgaeren et al. 2019). The genomic inflation factor was calculated as the median of the observed chi-squared test statistics divided by the expected median of the corresponding chi-squared distribution. Effect size of the significant variants (based on their odds ratio (OR)) and the minor allele frequency (MAF) (based on the frequency in this dataset) were plotted using GraphPad Prism 8.1.2 (GraphPad Software, La Jolla, California USA). For each variant, 0R and the 95% confidence interval (CI) was calculated in the total

population and the six subpopulations and plotted using GraphPad Prism 8.1.2 (GraphPad Software, La Jolla, California USA).

Logistic regression analysis was performed with stepwise backward modelling using R version 3.5.2 (R Foundation, Vienna, Austria). Calculation of linkage disequilibrium was performed using Haploview 4.2 (Broad Institute, Cambridge, MA, USA). First, linkage disequilibrium was calculated based on sequencing data from all cases and controls in this study. Second, data from the HapMap Project(Thorisson et al. 2005) was downloaded from the 1000 Genomes Project database(The Genomes Project 2015) and linkage disequilibrium was calculated for all markers in the region of the *ACAN* gene.

Gene-based tests included three implementations of the mutation burden test (Combined and Multivariate collapsing test (CMC), kernel-based adaptive cluster (KBAC) test, and the Variable Thresholds method (VT)), and two variance component analyses (SNP-set (Sequence) Kernel Association Test Method (SKAT) and the cAlpha test). All gene-based statistical tests were performed on variants reaching the MAF threshold of 0.01 in the control population, and on variants reaching the MAF of 0.001 in the control population. In addition, gene-based association tests were carried out stratified by variant type: 1) nonsense and frameshift variants, 2) nonsynonymous and in frame variants, 3) intronic variants, 4) 5'-UTR variants, 5) 3'-UTR variants.

Segregation of *ACAN* variants in otosclerosis families

Variants that were present in members of known otosclerosis families were checked in other family members. Variants of interest were resequenced using Sanger sequencing. Primers were developed using Primer3 software(Untergasser et al. 2012). PCR amplification was carried out under standard conditions. PCR products were sequenced on an ABI3130XL sequencer (Applied Biosystems Inc., Foster City, California, USA). Analysis of sequence data was done using the CLC DNA workbench 5.7.1 software (CLC bio, Aarhus, Denmark).

RESULTS

Exome sequencing results in independent variants in *ACAN*

Collection of cases and controls resulted in 1497 unrelated individuals with otosclerosis and 1437 ethnically matched control individuals with unknown otosclerosis status of Belgian, Dutch, French, Italian, Romanian and Swiss origin. Cases consisted of 1468 sporadic and 29 familial cases. For all individuals the entire coding region of *ACAN* and 5'- and 3'-UTR was sequenced.

To validate the NGS results, variants were compared to variants from the GnomAD v2 database to estimate the amount of false negative results. Around 90% of variants from the frequent variants (with a Minor Allele Frequency (MAF) > 0.01 in European, non-Finnish population) were present in our dataset. Furthermore, Sanger sequencing was carried out in 32 different samples, across different areas in *ACAN* covered by the smMIPs. In total, 6300 bases were Sanger sequenced. NGS variants were compared to Sanger sequencing, where the latter is considered to be the gold standard. Quality parameters were optimized to obtain a high positive predictive value (PPV), which indicates if the observed NGS variants are truly present. Under these quality parameters, we reached PPV of 98.45% (Supplementary Material and Methods).

To test the involvement of genetic variation in *ACAN* in otosclerosis, we carried out both single variant as well as gene-based association tests. Single variant association tests were carried out on all 426 variants detected in our sample set. The results showed 36 SNVs with a nominally significant association ($p<0.05$) and a lowest p-value of 1.76×10^{-17}. Two very rare variants occurred almost exclusively in cases (19 cases and 0 controls; and 19 cases and 1 control) showing p-values of 3.87×10^{-6} and 3.99×10^{-5} respectively. Interestingly, the rare alleles from the associated variants are not consistently enriched in cases. For some variants the rare allele is more frequent in controls, suggesting a protective effect of the rare allele. The distribution of the p-values in ACAN is shown as a QQ plot (Figure 1), which clearly shows an enrichment in significant p-values. Under the null hypothesis that none of the variants is associated, one would expect 21 SNVs to have a p-value below

0.05, whereas in our data, 36 SNVs showed a nominally significant association ($p<0.05$). False-discovery rate analysis showed that 14 of the SNVs had a q-value below 0.05. This means that, when declaring these 14 SNVs significant (Table 1), 95% of these are expected to represent a genuine association signal. All exonic SNVs are located in exon 12. One intronic SNV is found close to exon 6 (Figure 2)

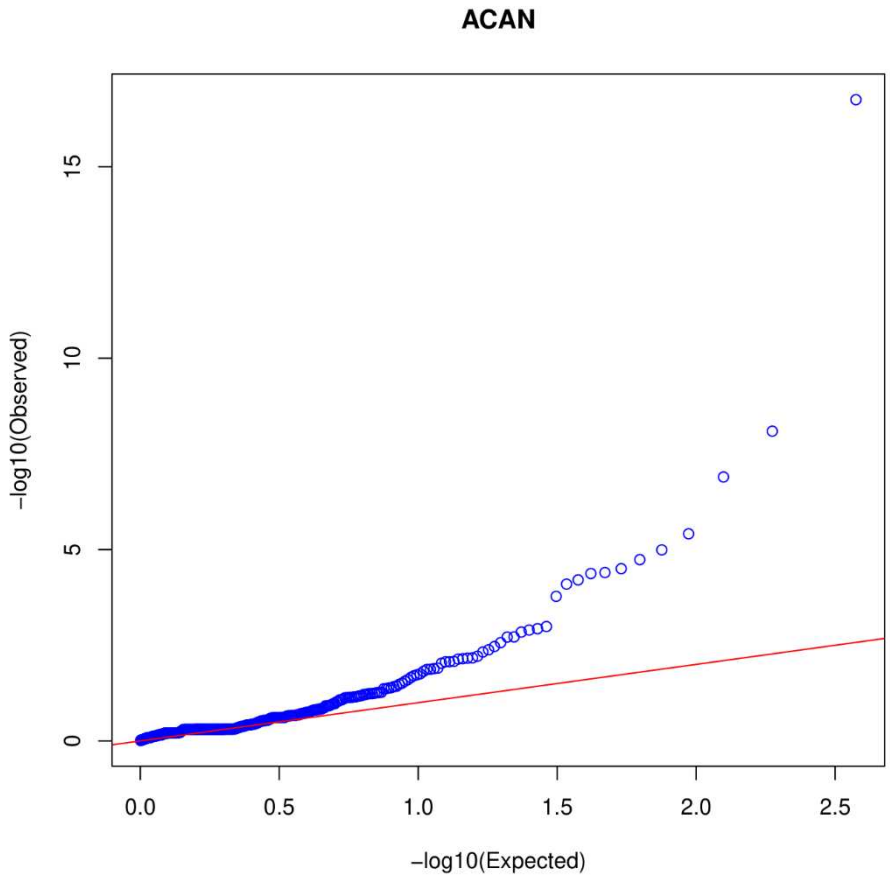

Fig. 1 QQ plot of the variants found in ACAN

The diagonal line shows the expected null distribution of the p-values in the absence of any association. The blue dots represent the observed p-values. The plot shows a clear enrichment of significant p-values compared to the null distribution (line).

Table 1 Overview of 14 significant variants in ACAN after false-discovery rate analysis

The table contains for each variant the chromosomal position with the genomic change, the number of alternative alleles and the number of total alleles in cases and controls, cDNA and amino acid change of the variant, the p-value and the allelic odds ratio. Genomic positions are according to NC_000015.9(ACAN) and cDNA positions according to NM_013227.3. Logistic regression with stepwise backward modeling showed 10 independent signals.

Variant	Genomic position	Variant alleles in cases / Total alleles in cases	Variant alleles in controls / Total alleles in controls	cDNA change	Amino acid change	P-value	Allelic odds ratio
1	g.89386527G>C	195/2894	257/2846	c.758-59G>C	p.(=)	1.42E-03	0.728
2	g.89398718T>C	4/2930	35/2852	c.2902T>C	p.(Ser968Pro)	1.26E-07	0.110
3	g.89398728G>A	1/2928	17/2852	c.2912G>A	p.(Gly971Glu)	6.20E-05	0.057
4	g.89398729A>G	103/2928	252/2850	c.2913A>G	p.(=)	1.76E-17	0.376
5	g.89398733C>A	1/2936	18/2852	c.2917C>A	p.(Leu973Ile)	3.14E-05	0.054
6	g.89400135C>A	6/2900	27/2846	c.4319C>A	p.(Thr1440Asn)	1.67E-04	0.216
7	g.89400147G>C	433/2862	343/2822	c.4331G>C	p.(Gly1444Ala)	1.17E-03	1.288
8	g.89400154G>T	252/2900	346/2846	c.4338G>T	p.(Glu1446Asp)	1.83E-05	0.688
9	g.89400205G>A	79/2870	163/2794	c.4389G>A	p.(=)	8.09E-09	0.457
10	g.89400224C>A	102/2912	52/2848	c.4408C>A	p.(Leu1470Ile)	7.99E-05	1.952
11	g.89400572C>A	19/2948	0/2854	c.4756C>A	p.(Leu1586Ile)	3.87E-06	∞

12	g.89401199C>G	77/2910	133/2850	c.5383C>G	p.(Leu1795Val)	4.24E-05	0.555
13	g.89401208C>G	80/2908	124/2850	c.5392C>G	p.(Gln1798Glu)	1.02E-03	0.622
14	g.89401239C>A	19/2920	1/2850	c.5423C>A	p.(Ala1808Glu)	3.99E-05	18.759

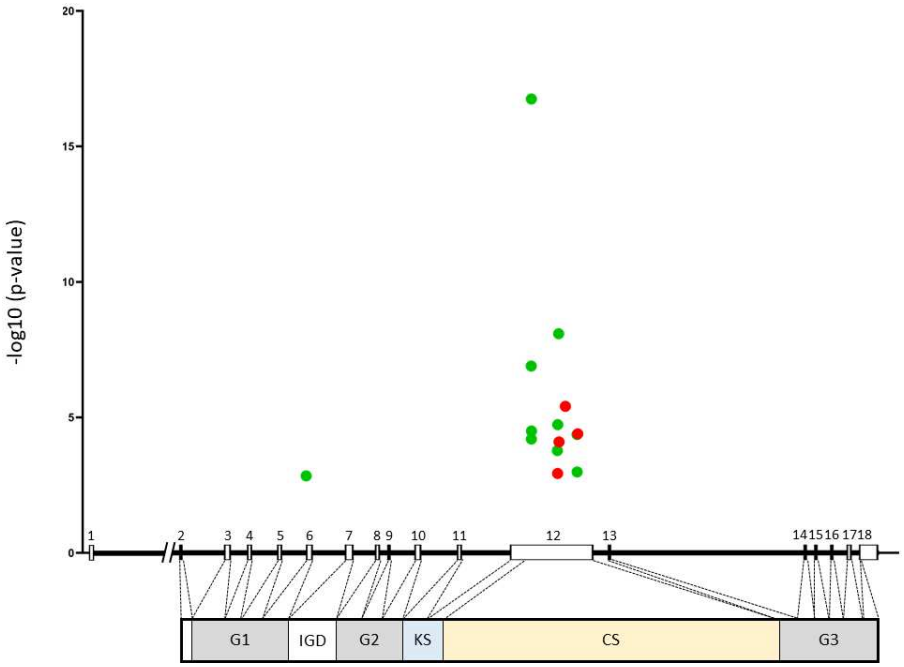

Fig. 2 Location of the variants in *ACAN*

The graph shows the -log10(p-value) of each variant and its location in the *ACAN* gene. The aggrecan protein consists of three globular domains (G1, G2 and G3), an interglobular domain between G1 and G2 (IGD), a keratan attachment domain (KS) and a large chondroitin sulfate attachment (CS) domain. Variant 1 is intronic and lies close to exon 6. All exonic variants cluster within the CS domain in exon 12. Variants where the rare allele is more frequent in the cases are indicated in red, whereas variants with the rare allele occurring more in controls are indicated in green

To investigate if the effect sizes of the 14 associated SNVs were consistent across the 6 subpopulations from our study population we plotted the allelic Odds Ratio (OR) and 95% confidence interval for each subpopulation and the total population (Figure 3). Consistent association was reached when all OR of all subpopulations were lower (or higher) than 1. Six out of 14 variants (2, 3, 5, 6, 11 and 14 (Table 1)) show a clearly consistent association, with all ORs being either larger than 1, or smaller than 1. Four out of 14 variants (1, 4, 9 and 12 (Table 1)) showed a near consistent association.

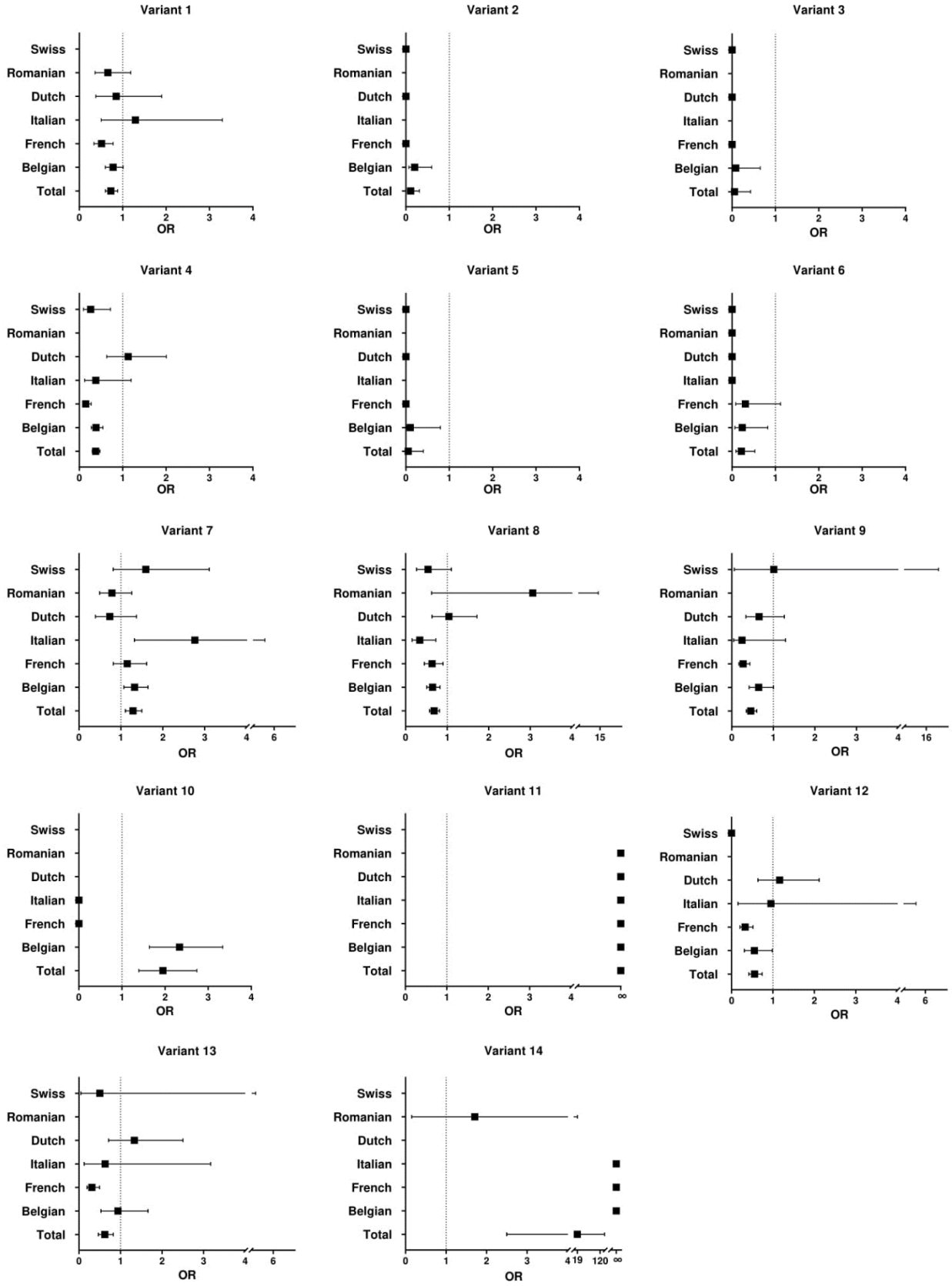
Variant 1
Variant 2
Variant 3
Variant 4
Variant 5
Variant 6
Variant 7
Variant 8
Variant 9
Variant 10
Variant 11
Variant 12
Variant 13
Variant 14
Swiss
Romanian
Dutch
Italian
French
Belgian
Total
OR

Fig. 3 Forrest plots showing odds ratios from all subpopulation and the total population

For each variant in the total population and in each subpopulation, the point estimate of the odds ratio (OR) is represented by a square, and the 95% confidence interval (CI) by a horizontal line. In case the variant was only observed in cases, but not observed in controls, OR was set to ∞. In case the variant was not observed in cases, OR was set to 0. In these two extreme cases, the CI could not be calculated. The vertical line shows the value of the OR in case of no association. A variant is consistent across the subpopulations if all OR estimates are at the same side of this vertical line. The plots show a (near) consistent association for 10 out of 14 variants across the different subpopulations.

To test for a possible inflation of p-values due to population stratification, the genomic inflation factor was calculated, based upon the test statistics observed in a previous study (Valgaeren et al. 2019) using the same samples, where no association with otosclerosis was observed. This study showed no indication for population stratification, with genomic inflation factors around 1 (results not shown). This suggests that spurious associations due to population stratification are unlikely in the current data.

The multitude of significantly associated SNVs in *ACAN* could indicate many independent variants all having an effect on disease risk or could instead reflect one underlying association signal with surrounding SNVs significantly associated to the phenotype due to high linkage disequilibrium (LD) with the causative SNV. To discern between these two scenarios, we first investigated the LD in the *ACAN* region using the 1000 Genomes data, and the LD structure between the SNVs found in this study (Figure S1). None of these indicated a strong LD in and around the *ACAN* gene, favoring the hypothesis of independent signals. In addition, we carried out conditional logistic regression analysis. Starting from a regression model including all 14 significant SNVs, stepwise backward elimination resulted in a model with 10 SNVs still being significant. This result was in line with the study of linkage disequilibrium within the *ACAN* gene, that showed very little LD between the *ACAN* variants, and implicated that *ACAN* contains many independent association signals.

Similar to the figure on genetic architecture of complex diseases by Manolio and colleagues (Manolio et al. 2009), effect size (based on odds ratio) of the 14 significant SNVs and the MAF (based on the

frequency in this dataset) were plotted (Figure 4). The plot shows a broad spectrum of variants, ranging from rare variants with high effect sizes to more common variants with low effect size.

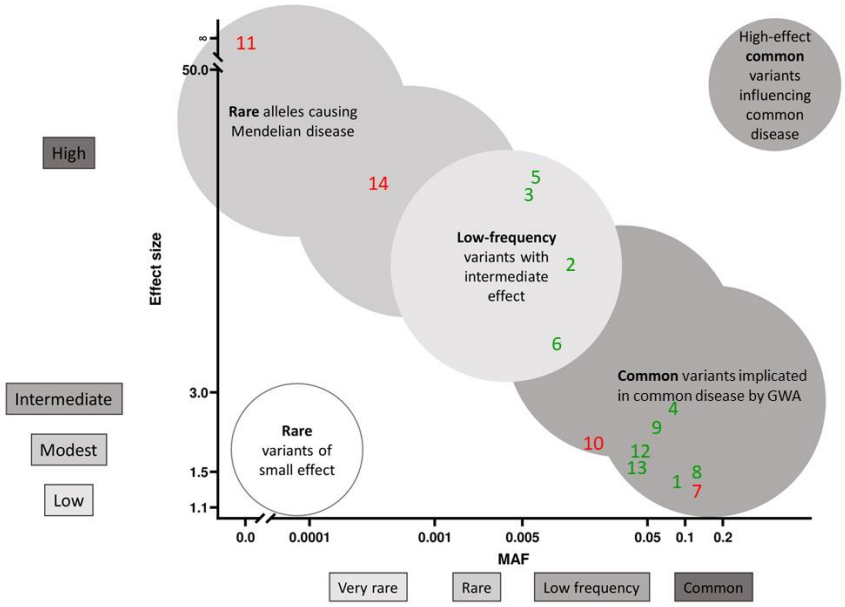

Fig. 4 Plot of the minor allele frequency (MAF) and the effect size (based on the odds ratio)

The plot shows a spectrum ranging from rare variants with a large effect size to common variants with a small effect. Manolio et al. (Manolio et al. 2009) discussed this particular variant spectrum profile for the first time to describe the genetic architecture underlying complex diseases. Otosclerosis occurs both as a familial as well as a sporadic disorder, indicating that different variants with different frequencies are responsible. Here, we found several variants in ACAN that reflect this complex spectrum. In addition, our results show both predisposing (circles) and protective (stars) variants that are associated with the disease.

Gene-based tests reveal cumulative effect of (very) rare variants

The single variant association tests based upon Fisher's exact test, only have sufficient power if the variant is present at intermediate allele frequencies in the data. Very rare variants, only occurring a few times offer no statistical power to test for the association between the very rare allele and the phenotype. In order to test the cumulative effect of multiple rare variants on the phenotype, several gene-based tests were carried out under a wide range of models and assumptions, stratifying for allele frequency and for variant type (Table 2).

Table 2. Result of gene-based tests that were carried out under a wide range of models and assumptions, stratifying for variant type.

Gene-based tests included three mutation burden tests (Combined and Multivariate collapsing test (CMC), kernel-based adaptive cluster (KBAC) test, and the Variable Thresholds method (VT)), and two variance component analyses (SNP-set (Sequence) Kernel Association Test Method (SKAT) and the cAlpha test). Variant type "All" represents all very rare variants with a minor allele frequency (MAF) smaller than 0.001 and shows significant associations between the phenotype and the cumulated very rare variants in both the burden tests and the variance component-based tests. All other variant types are the results of stratifying for variant type and show significant and consistent associations of the phenotype with the very rare nonsynonymous variants and with the very rare variants in the 3'-untranslated region (UTR).

Variant type	Mutation burden tests			Variance component analysis	
	CMC	KBAC	VT	SKAT	cAlpha
All	0.0055	0.0064	0.022	0.00012	0.034
Intronic	0.27	0.93	0.82	0.82	0.92
Non-synonymous & Frameshift	0.00069	0.0012	0.0022	4.6x10-5	0.0016
3'-UTR	0.029	0.026	0.041	0.89	0.015
5'-UTR	1.0	0.41	0.70	0.42	0.50

When stratifying only for allele frequency (lumping all variant types), consistently significant associations were found between the phenotype and the cumulated very rare variants, and between the rare variants and the variance component-based tests. When we additionally stratify for variant type, highly significant and consistent associations were found with the very rare nonsynonymous variants, and with the very rare variants in the 3'-UTR. The highly significant associations for the very rare nonsynonymous variants were entirely attributable to two variants already detected through the single variant tests (variant 11 and 14). When removing these two variants from the gene-based analysis, the association with the nonsynonymous very rare variants was no longer significant. The significant association with the very rare variants in the 3'-UTR, however, remained. None of the very rare 3'-UTR variants appeared in the data more than two times and none of them had a significant p-

value upon single variant testing, but when aggregating their effects the association with the phenotype was significant in 4 out of 5 association tests we performed.

Segregation of variants in otosclerosis families

Variants 11 and 14 were identified in 19 cases each. Variant 14 was identified in one control whereas variant 11 was absent in controls. Five and three familial cases had variant 11 and 14, respectively, suggesting that these two variants could cause monogenic otosclerosis with reduced penetrance. Further identification of the familial cases showed that the three cases with variant 14 also carried variant 11. From these five individuals, three did not have any additional family members available for testing. Therefore, we performed segregation analyses using Sanger sequencing in two families, where additional family members were available. In both families, the variant did not cosegregate with the disease (results not shown).

DISCUSSION

In this study, targeted parallel sequencing was used to study the importance of the *ACAN* gene in the occurrence of otosclerosis. Our results show 14 highly significant variants associated with the phenotype, of which eight showed a consistent association across six subpopulations. The rare allele of four variants is more frequent in cases, whereas the rare allele of the other ten variants is more frequent in controls. This suggests that variants in *ACAN* act as either protective or predisposing variants. They also reflect a wide spectrum of variants ranging from very rare variants with a large effect, to common variants with a small effect (Figure 4). Manolio et al. (Manolio et al. 2009) discussed this particular variant spectrum profile for the first time as an explanation for the genetic architecture of complex diseases. Similar graphs have been reported previously (Allin et al. 2014; Hindorff et al. 2011). However, for most traits and diseases, this full spectrum of frequencies and effect sizes is only reached when multiple genes are involved. The current study is, to our knowledge, the first one where both protective and predisposing alleles in one single gene represent the entire spectrum of frequencies and effect sizes. The mutational spectrum we report includes a number of very rare

variants with high effect sizes. Such variants with a large effect on the encoded protein normally result in monogenic diseases. Here, despite their high effect size, cosegregation was not observed in families. As there were only two families available for testing, this does not exclude these variants as a possible cause of familial otosclerosis because protective variants in ACAN or other genes may reduce the penetrance (Moumoulidis et al. 2007). Variants at the other end of the spectrum, which are neither necessary nor sufficient to result in a disease state, typically underlie complex traits and diseases. The cumulative effect of multiple variants in multiple genes, combined with environmental risk factors results in the disease state. The reported variation in *ACAN* is in line with the occurrence of both sporadic and familial forms of otosclerosis.

Missense variants, nonsense variants and frameshift variants in aggrecan cause bone diseases, such as Kimberley type spondyloepiphyseal dysplasia, and isolated short stature with or without advanced bone age, early-onset osteoarthritis and/or osteochondritis dissecans (Nilsson et al. 2014; Stattin et al. 2010; Stavber et al. 2020). *Acan* knockout mice have skeletal growth plates with disorganized chondrocytes closely packed with reduced ECM resulting in perinatal lethal dwarfism and craniofacial abnormalities (Watanabe et al. 1994). Remarkably, all exonic variants we found, are located within the CS domain of the aggrecan protein (Figure 2). Previously only two other missense variants in this domain have been reported causing an autosomal recessive form of skeletal dysplasia called aggrecan type spondyloepimetaphyseal dysplasia in a 45-year old patient (Fukuhara et al. 2019). These variants are different from the variants found in the current study, which suggests that variants in the CS domain result in different diseases compared to variants in the other domains.

In a recent paper, where pathogenic variants in *ACAN* were observed in 19 patients with short stature, one person was reported to have both otosclerosis and short stature (Stavber et al. 2020). If we calculate the observed prevalence in this paper (1 otosclerosis case in 19 individuals, or 0.0526) and the 95% confidence interval (ranging from 0 to 0.155), we see that the prevalence of otosclerosis in the general European population (0.3 – 0.4% (Declau et al. 2001)) lies within this interval. This means

that there is no indication that otosclerosis is more prevalent amongst patients with short stature. In addition, as the number of patients was too small and included patients younger than the age of onset of otosclerosis, it is not possible to draw any conclusion about this co-occurrence. The common denominator among all these previous publications is that variants in aggrecan influence the normal cartilage structure and bone formation, albeit in different tissues.

Gene-based tests showed two consistent association signals across a wide range of models: very rare nonsynonymous (5/5 tests) and 3'-UTR variants (4/5 tests). The former signal was entirely attributable to variants 11 and 14. When omitting these two variants, no significant signals were found. Yet, this result could serve as a positive control that a gene-based test shows a significant association in the presence of rare alleles strongly associated with the phenotype. On the other hand, the association signal observed for the variants in the 3'-UTR could not be attributed to one or a few single variants. This suggest that a cumulative effect of very rare variants in the 3'-UTR has an effect, while none of the single variants within the 3'-UTR were independently associated with otosclerosis. This finding is in line with the important role of non-coding low-frequency alleles in phenotypic variants, as described in the UK10K study (Consortium et al. 2015), where a strong enrichment of variants with lower effect sizes was discovered in non-coding domains, including 3'-UTRs. The effects of 3'-UTR variants have been shown to be tissue-specific and because of their influence on gene expression, mRNA export, cytoplasmatic localization and stability, they could play a role in complex diseases (Mariella et al. 2019). Specifically in aggrecan, the 5'-UTR strongly stimulates the expression, whereas the 3'-UTR inhibits the activities of the promotor (Valhmu et al. 1998). The effect of the 3'-UTR has been shown to be tissue specific. Presence of 3'-UTR shows a significant suppression of the aggrecan promotor in chondrocytes, but not in fibroblasts. Therefore, it is tempting to speculate that the position of variants in *ACAN* and the variants in the 3'- UTR, determine the affected tissue. Variants in the 3'-UTR will suppress aggrecan more in some tissues, thus altering the disease outcome. This could explain why, according to our knowledge and a PubMed literature search, there are no clear indications that

otosclerosis is more prevalent amongst patients with *ACAN* mutations that cause other bone disorders.

Taken together, our results show an association of the *ACAN* gene with otosclerosis. However, the exact pathway and the role in the pathogenesis of otosclerosis remains unclear. Previous studies have also indicated a role for *ACAN* in hearing disorders in human (Dawson et al. 2018; Hoffmann et al. 2016a; Mangino et al. 2001). In addition mice with mutant aggrecan show hearing loss (Yoo et al. 1991), proving the importance of normal aggrecan expression in hearing. A possible explanation could be the role of aggrecan in the normal development of the ossicles and the inner ear. The ossicles are formed through endochondral ossification, where aggrecan expression plays a role in the differentiation of chondrocytes (Chapman 2011; Ovadia et al. 1980). Studies on the development of the inner ear have also shown expression of *ACAN* during the development of the otic capsule. (Ficker et al. 2004). An decrease of aggrecan expression has been associated with disrupted chondrification of the otic capsule (Bast 1933; Chapman 2011). However, bone growth and remodeling in the middle and inner ear are mediated by several pathways, of which *ACAN* is only one part of the puzzle. Apart from *ACAN,* several other bone morphogenetic genes have been associated with otosclerosis, such as *TGFB1, BMP2* and *BMP4* (Schrauwen et al. 2008). In addition, a recent GWAS confirmed association of the *TGFB1* pathway with otosclerosis, again suggesting the involvement of these bone morphogenetic genes (Rämö et al. 2020).

Here, we have presented important results in the genetics of otosclerosis. However, this study has a few limitations. First, we only sequenced the coding region, 5'- and 3'-UTR of the *ACAN* gene. The smMIPs had a 10 bp overhang of the exon-intron boundaries. In these overhangs we found 1 highly significant variant close to exon 6 (Table 1 and Figure 2). Additional sequencing of the full gene might lead to identification of more intronic variants. Second, we focused on one candidate gene, as previous studies had indicated, *ACAN* to be a promising candidate gene for otosclerosis. However, otosclerosis is a complex disease caused by multiple genes and environmental factors. Studies into the effect of

multiple genes could give more insight into the genetic architecture of the disease. Lastly, all exonic variants found in this study where located in the CS domain of aggrecan. Future functional studies are needed to investigate the impact of these variants on the protein and on the pathogenesis of otosclerosis.

In conclusion, our study illustrates an important role for *ACAN* in the onset of otosclerosis. These results are quite extraordinary, with a single gene containing multiple variants of both protective and predisposing nature, ranging from high frequency with low effect size, to very high effect size with very low frequency. To our knowledge, these findings are unique in the field of complex genetics. Most of the significant variants identified in this study have more extreme odds ratios with higher odds ratios for predisposing and lower odds ratios for protective variants than reported in other genes associated with otosclerosis (Table 1 and Table S1). In addition, we observed an important cumulative effect of very rare variants in the 3'-UTR. Together these findings nicely explain the genetic characteristics of otosclerosis.

ACKNOWLEDGEMENTS

We would like to acknowledge Robert Vincent (Causse Ear Clinic, France) and Marcel Cosgarea (Iuliu Hatieganu University of Medicine and Pharmacy, Romania) for providing patients for this study. This study was supported by funding from the Belgian Science Policy Office Interuniversity Attraction Poles (BELSPO-IAP) program (project IAP P7/43-BeMGI to G.V.C.) and the Obelske Familiefond (to A.T.H. and M.B.P.).

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