Human Molecular Genetics, 2008, Vol. 17, No. 2 ***159–169***
doi:10.1093/hmg/ddm292
Advance Access published on October 6, 2007

The grainyhead like 2 gene (*GRHL2*), alias *TFCP2L3*, is associated with age-related hearing impairment

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Received September 13, 2007; Revised and Accepted October 1, 2007

Age-related hearing impairment (ARHI) is the most prevalent sensory impairment in the elderly. ARHI is a complex disease caused by an interaction between environmental and genetic factors. The contribution of various environmental factors has been relatively extensively studied. In contrast, investigations to identify the genetic risk factors have only recently been initiated. In this paper we describe the results of an association study performed on 2418 ARHI samples derived from nine centers from seven European countries. In 70 candidate genes, a total of 768 tag single nucleotide polymorphisms (SNPs) were selected based on HAPMAP data. These genes were chosen among the monogenic hearing loss genes identified in mice and men in addition to several strong functional candidates. After genotyping and data polishing, statistical analysis of all samples combined resulted in a *P*-value that survived correction for multiple testing for one SNP in the *GRHL2* gene. Other SNPs in this gene were also associated, albeit to a lesser degree. Subsequently, an analysis of the most significant *GRHL2* SNP was performed separately for each center. The direction of the association was identical in all nine centers. Two centers showed significant associations and a third center showed a trend towards significance. Subsequent fine mapping of this locus demonstrated that the majority of the associated SNPs reside in intron 1. We hypothesize that the causative variant may change the expression levels of a *GRHL2* isoform.

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INTRODUCTION

Age-related hearing impairment (ARHI), or presbyacusis, is the most common sensory impairment seen in the elderly. Approximately 60% of people aged between 71 and 80 suffer from an impairment of 25 dB or more (1). As the overall population in developed countries is aging, the number of ARHI subjects is expected to increase steadily during the coming years.

ARHI is a complex disorder with both environmental and genetic factors contributing to the sensory deficit as indicated by several heritability studies (2–5). Heritability estimates ranged between 0.25 and 0.75 depending on the study design (family versus twin study), the frequency range (low versus high frequencies), the age range (below 65 or above), the pathological type (strial versus sensory ARHI) and the method of measuring hearing (self-reported hearing impairment versus pure tone or speech audiometric measurements).

The impact of several environmental risk factors has been investigated extensively. These include noise exposure (6–8), ototoxic medication (9,10), and exposure to chemicals (11,12). Certain medical conditions such as diabetes (13), cardiovascular disease (14) and renal failure (15) may also have an impact on ARHI. The effects of nutritional status (16–18), tobacco smoking (9,19,20), alcohol (ab)use (9,19,21) and bone mass density (22–24) remain controversial.

Less is known concerning the nature of the genetic factors involved in the development of ARHI as these have only recently attracted the attention of scientists. So far, two genome wide linkage studies have been performed, resulting in the localization of seven different susceptibility regions for ARHI (25,26). In addition, a number of association studies on candidate susceptibility genes have been performed. Three of these failed to detect significant associations with ARHI (27–29). Other studies succeeded in identifying genes that are associated with ARHI. A significant association was found between ARHI and a polymorphism in N-acetyltransferase (*NAT2*), an enzyme involved in the metabolism and detoxification of cytotoxic and carcinogenic compounds (30). We recently replicated this finding in an independent population (31). In the latter study we also found evidence for an association of the *GSTM1* (glutathione s-transferase, mu-1) and *GSTT1* (glutathione s-transferase, theta-1) deletion polymorphisms and ARHI in the Finnish population (31). In addition, we previously demonstrated that ARHI was associated with several SNPs in a 13 kb region in *KCNQ4* (potassium channel, voltage-gated, kqt-like subfamily, member 4) (32). Finally, an apparent protective effect of apolipoprotein E (APOE) allele $\varepsilon 4$ was recently suggested (33).

Although whole genome association studies are feasible nowadays, they remain very expensive. Therefore, to identify susceptibility genes involved in complex disorders, a candidate gene approach is often pursued. In the case of ARHI, a whole range of candidate genes can be proposed as the perception of sound requires complex pathways and age-related changes in any component of these pathways may contribute to ARHI. Genes causing monogenic forms of hearing loss are excellent candidate susceptibility genes for ARHI. Other genes can be considered candidates on the basis of a known or presumed function in the inner ear. Here we present the results of an association study on 70 candidate genes, which were chosen among the monogenic hearing loss genes identified in mice and men in addition to several strong functional candidates. In this study we used 2418 ARHI samples derived from nine centers from seven European countries.

RESULTS

Results of the genotyping project and data polishing

The 34% extremes from 3530 samples were selected for both genders and for each center separately (Table 1 and Supplementary Material, Fig. S1). Two thousand four hundred and fifty four selected samples (including 36 duplicate samples) were genotyped for 768 SNPs selected from 70 candidate genes (Tables 2, 3 and Supplementary Material, Table S1). An overview of the success rates of the genotyping project is given in Table 4. The overall success rate was 92.93%. Moreover, the Illumina genotyping method gave highly reproducible results (Table 4). Checking 3432 of the Illumina genotypes with an alternative genotyping method in our laboratory setting, resulted in an accuracy of 99.94%. The subsequent removal of genetic outliers based on CHECKHET and GRR (graphical representation of relationship errors) resulted in a final number of 2318 samples for which results were suitable for statistical analysis (Table 1).

Association analysis

Although the minor allele frequency (MAF) cut-off value was set at 0.05 during SNP selection based on HAPMAP data, 19 SNPs were below that cut-off value when genotyped on our sample sets. Hence, despite the fact that Illumina had successfully genotyped 722 SNPs, only 703 were taken forward for statistical analysis. As a first step, the homogeneity of the genotyping results across the different centers was tested using logistic regression. If this test was not significant, the samples of all centers were combined and a reduced logistic regression model was fitted to analyze the effect of the genotypes on the ARHI phenotype. Table 5 lists the 20 top-ranked SNPs resulting from the combined analysis. When we applied a Bonferroni correction for multiple testing ($0.05/703 = 7.1 \times 10^{-5}$), none of the *P*-values remained significant. However, the top-ranked SNP, rs10955255 in *GRHL2 (grainyhead like 2)*, almost reached this Bonferroni-corrected significance level (8.4×10^{-5}). Since not all 703 tests were independent because of linkage disequilibrium (LD) between SNPs, a Bonferroni correction for multiple testing can be considered too conservative. Moreover, false discovery rate (FDR) calculations resulted in a Q-value of 0.054, which indicates that the probability of this association being spurious was only 5%. Interestingly, all the three top-ranked SNPs resided in *GRHL2* in a region of approximately 16 kb. These three *GRHL2* SNPs were in LD (data not shown). The FDR Q-value for the third-ranked SNP (rs1981361) was 0.081, indicating that among the three top-ranked SNPs, only 8% were expected to be false discoveries. Taking everything into account, we concluded that the *GRHL2* result represents a genuine association.

Table 1. Overview of sample numbers

Country	Center	Number of samples collected	Number of women selected	Number of men selected	Total selected	Total removed[a]	Available for analyses
Belgium	Antwerp	769	276	248	524	19	505
United Kingdom	Cardiff	368	112	140	252	23	229
Denmark	Copenhagen	404	138	140	278	9	269
Belgium	Ghent	198	76	60	136	5	131
The Netherlands	Nijmegen	276	88	100	188	4	184
Finland	Oulu	505	196	150	346	11	335
Italy	Padova	359	130	116	246	13	233
Finland	Tampere	256	106	70	176	5	171
Germany	Tübingen	395	134	138	272	11	261
Total		3530	1256	1162	2418	100	2318

[a]This number includes samples that failed genotyping, those with a gender mismatch and genetic outliers detected by CHECKHET or GRR.

Table 2. Classification of candidate genes

Category	Number of genes
Non-syndromic[a]	36
Functional	14
Mouse	8
Oxidative stress	5
Syndromic	3
Expression	2
Y-chromosome	1
Stratification	1
Total	70

[a]Genes leading to both syndromic and non-syndromic hearing loss were classified as non-syndromic genes.

Table 3. Classification of selected SNPs

Category	Number of SNPs	Percentage (%)
Intronic	623	81.1
Exon/non-synonymous	61	7.9
Exon/synonymous	13	1.7
UTR	25	3.3
Locus	46	6.0
Total	768	100.0

Ten from the 20 top-ranked SNPs were localized in *PCDH15* (protocadherin 15). Because the Bonferroni-corrected significance limit was never reached for the combined analysis of the 10 *PCDH15* top-ranked SNPs (a minimum *P*-value of 0.0015 was obtained; Table 5), and because FDR Q-values of 0.249 and higher were calculated, this lead was not pursued.

Next, the directions of the associations were analyzed for all SNPs in all centers separately. For only three out of 703 SNPs, all odds ratios (ORs) pointed in the same direction: *GRHL2* SNPs rs10955255 and rs2127034 (ranked 1 and 2, respectively) and *PCDH15* SNP rs7087057 (ranked 11). This fact provided further evidence for the validity of the association between *GRHL2* SNPs rs10955255 and rs2127034 and ARHI.

For *GRHL2* SNP rs10955255, significant associations were observed for the centers of Oulu and Padova (*P*-values of 0.0020 and 0.0036, respectively; Table 6), while for Tübingen a trend towards significance was observed (*P*-value = 0.082; Table 6). ORs ranged between 1.02 and 1.76. For all populations except Ghent, the GG genotype was more often present in subjects with worse hearing than in better hearing subjects. The AA genotype was protective as it was more frequent in better hearing subjects than in worse hearing subjects (Fig. 1). For *GRHL2* SNP rs2127034, the Oulu subsample was significantly associated with ARHI (*P*-value = 0.0009; Table 6) and ORs ranged between 0.58 and 0.92. Subsequently, the association between ARHI and variations in *GRHL2* was further elaborated.

GRHL2 fine mapping

For fine mapping purposes 44 additional tag-SNPs and a non-synonymous SNP located in exon 2 (rs3735709) were genotyped. A sample success rate of 95.3% (114 samples missing from 2418), a SNP success rate of 91.1% (41 SNPs from 45) and an overall success rate of 86.8% were obtained.

The results of the statistical analysis of the 26 original and the 41 fine-mapping *GRHL2* SNPs on all samples combined are shown in Table 7 and in Supplementary Material, Table S2. Besides the three top SNPs from the first analysis (rs10955255, rs2127034 and rs1981361), one additional SNP (rs13263539) was highly significantly associated with ARHI with a *P*-value of 0.0002. In addition, six SNPs were associated with *P*-values ranging between 0.01 and 0.04. The non-synonymous SNP in exon 2 (rs3735709), which was located in close proximity of SNPs rs10955255 and rs2127034 and as such a putative causative SNP, was not significantly associated with ARHI.

Subsequently, we investigated the LD patterns within *GRHL2* with Haploview. In addition, we looked for putative regulatory regions with Genomatix (Fig. 2). The four highly significant SNPs and two of the less significant SNPs all resided in one 18 kb LD-block located in the second half of intron 1. One of the predicted promoter regions was located within this LD-block (Fig. 2), and it also contained a few regions that were conserved between man and mouse (Supplementary Material, Fig. S2). The remaining four less significant SNPs were present in the last part of the gene. SNPs rs812890 and rs12114698 reside in the associated 18 kb

Table 4. Summary of the results of the genotyping project

Parameter	Number of successful genotypes	Number of possible genotypes	Success rate (%)
Sample success rate[a]	2429	2454	98.98
Locus success rate[b]	722	768	94.01
Genotypes (call rate)[c]	1745275[f]	1747547[f]	99.87
Reproducibility[d]	36	36	100
Overall success rate[e]	1745275[f]	1878019[f]	92.93

[a]Number of samples out of the total attempted for which genotypes were delivered.
[b]Number of assays designed for the multiplex analysis that were successful in producing genotypes at the desired loci.
[c]Number of genotypes delivered for all successful samples at all successful loci.
[d]Reproducibility of the replicate sample pairs.
[e]Number of genotypes delivered for all samples at all loci.
[f]Excludes Y-genotype calls on female samples.

Table 5. Top 20 of associated SNPs in all samples combined

Rank	Gene	SNP	Chr	Coordinate	MAF	Homogeneity	*P*-value	OR
1	*GRHL2*	rs10955255	8	102605581	0.400	0.2948	8.38×10^{-5}	1.27
2	*GRHL2*	rs2127034	8	102611574	0.467	0.5885	0.000160	0.80
3	*GRHL2*	rs1981361	8	102621953	0.404	0.0728	0.000529	0.81
4	*EYA4*	rs212765	6	133831944	0.460	0.3255	0.000565	1.23
5	*ITGA8*	rs2236579	10	15802120	0.168	0.8402	0.000606	0.76
6	*PCDH15*	rs7081730	10	55703027	0.283	0.6018	0.001507	1.23
7	*PCDH15*	rs11004270	10	55817364	0.255	0.6827	0.001698	0.80
8	*KCNQ1*	rs12277647	11	2420601	0.301	0.7407	0.001710	1.22
9	*KCNMA1*	rs697173	10	78883639	0.378	0.7568	0.001967	0.83
10	*PCDH15*	rs996320	10	55671084	0.279	0.7127	0.001983	1.23
11	*PCDH15*	rs7087057	10	55682161	0.26	0.8476	0.002025	0.81
12	*PCDH15*	rs978842	10	55735045	0.269	0.4529	0.002579	0.81
13	*PCDH15*	rs7476518	10	55661120	0.278	0.7054	0.004174	1.21
14	*ITGA8*	rs1417664	10	15778660	0.292	0.2526	0.005117	1.20
15	*PCDH15*	rs1900443	10	55278131	0.143	0.0704	0.006731	1.25
16	*PCDH15*	rs10509013	10	55957107	0.229	0.1335	0.007234	1.21
17	*CAT*	rs2300181	11	34433115	0.230	0.8105	0.008213	1.21
18	*PCDH15*	rs11004142	10	55642037	0.343	0.7360	0.008259	0.85
19	*PCDH15*	rs12258253	10	55298564	0.142	0.6267	0.014513	0.81
20	*EYA4*	rs9321402	6	133836204	0.299	0.4520	0.016562	0.86

Chr, chromosome; MAF, minor allele frequency; OR, odds ratio.

Table 6. Association analysis of the two most significant *GRHL2* SNPs in all centers separately

Center	rs10955255		rs2127034	
	OR	*P*-value	OR	*P*-value
Antwerp	1.05	0.7199	0.92	0.4922
Cardiff	1.12	0.5706	0.88	0.4998
Copenhagen	1.28	0.1995	0.90	0.5481
Ghent	1.02	0.9421	0.82	0.3985
Nijmegen	1.17	0.4667	0.78	0.2352
Oulu	1.67	**0.0020**	0.58	**0.0009**
Padova	1.76	**0.0036**	0.73	0.0794
Tampere	1.22	0.4153	0.91	0.6881
Tübingen	1.36	0.0821	0.76	0.1056

Significant *P*-values have been indicated in bold.

LD-block, but were not significantly associated. This is most likely due to their low MAF of 0.018 and 0.014, respectively.

To test whether all significant SNPs were attributable to the same association signal, we performed an allelic heterogeneity test. With this analysis we investigated whether LD with the most significant SNP (rs10955255) accounted for all other associated SNPs. The results showed that all associated SNPs residing in the 18 kb haplotype block represent a single association signal and, hence, the same underlying causative mutation (Supplementary Material, Table S3). From the associated SNPs in the distal part of the gene, all but one were attributable to that same association signal. Only SNP rs7827945 seems to be independent. This latter association was not very significant and may be coincidental.

Haploview enabled us to determine the risk haplotype in the 18 kb LD-block (Table 8). The most frequent haplotype, GCAATAGAG, is also the risk conferring haplotype with a *P*-value of 0.0003.

DISCUSSION

Our ARHI candidate gene study, consisting of the analysis of 768 SNPs selected in 70 genes, has identified *GRHL2*, alias *BOM* (brother of mammalian grainyhead) or *TFCP2L3*

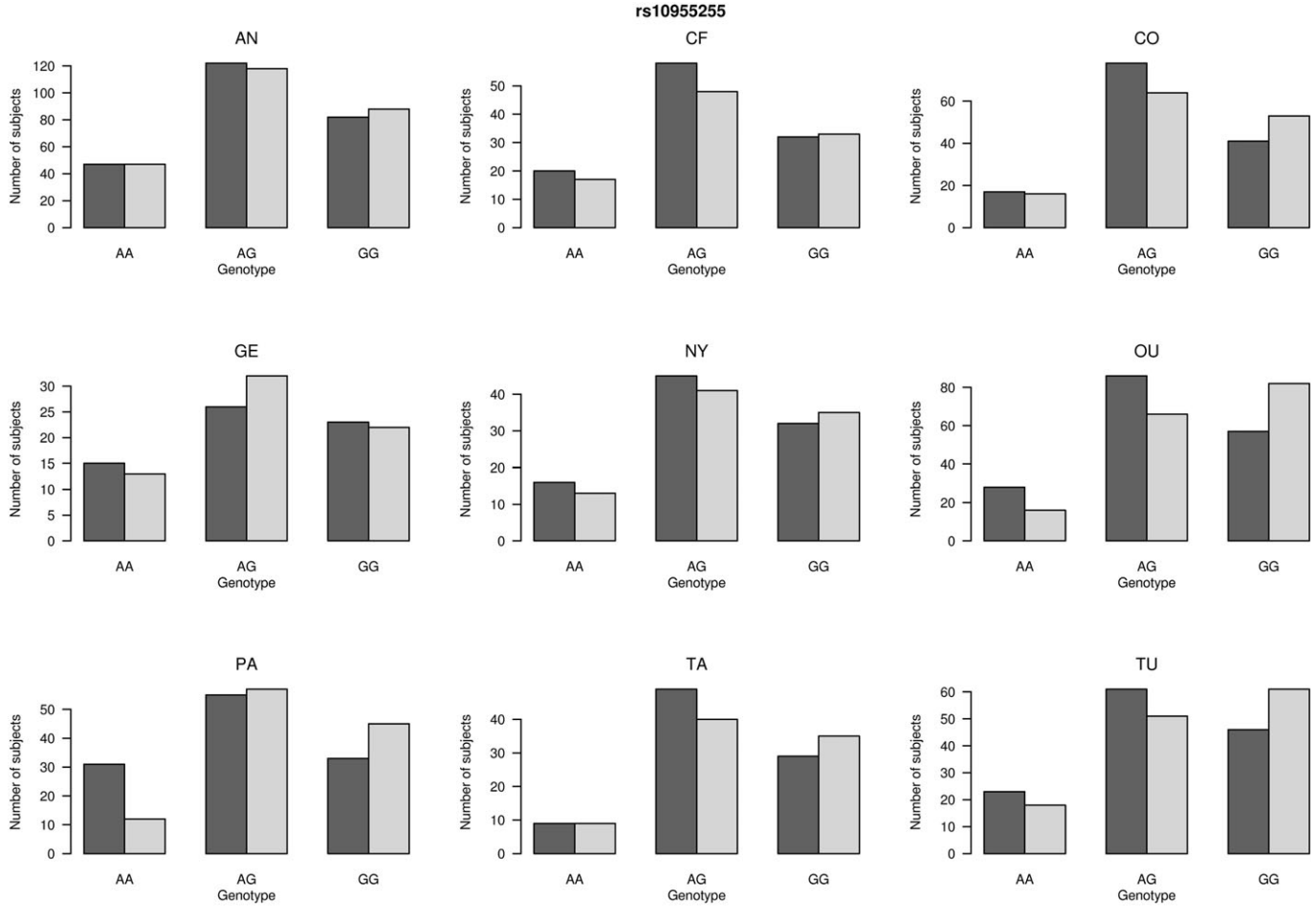

Figure 1. Barcharts of the *GRHL2* SNP rs10955255 genotypes for all subsample sets. Worse hearing subjects (cases) are indicated with a lighter shaded box, while better hearing subjects (controls) are indicated with a darker shaded box. X-axis refers to the genotypes, while the Y-axis refers to the number of subjects. AN, Antwerp; CF, Cardiff; CO, Copenhagen; GE, Ghent; NY, Nijmegen; OU, Oulu; PA, Padova; TA, Tampere and TU, Tübingen.

(transcription factor cellular promoter 2-like 3), as an ARHI susceptibility gene. This conclusion was based on several findings: four SNPs residing in one 18 kb LD-block were highly significantly associated with ARHI in a combined analysis of all samples, the most significant SNP was replicated in two out of nine independent sample sets, and the ORs of all nine sample sets pointed in the same direction. In addition, the GG-genotype was more prominent in worse hearing subjects than in better hearing subjects for all but one center (Ghent). Up to now, we have not been able to find a reasonable explanation for the discrepant picture obtained in the samples from Ghent. These samples have been collected using exactly the same criteria as for the other samples. In addition, Ghent is very close to Antwerp and Nijmegen geographically. The Ghent subsample contained the smallest number of samples, and was consequently the least powerful subset. As such, a plausible explanation might be that these results deviate from the others simply by chance.

A preliminary analysis of audiogram shapes has indicated that the Ghent subsample has a higher proportion of flat-shaped audiograms, while in most of the other subsamples the high-frequency steeply sloping audiogram shape is the most prominent (unpublished data). Inherent on the *Z*-score method, a subject with a flat audiogram is more likely to be included among the controls. Interestingly, Ghent had a relatively higher proportion of cases with a flat audiogram and an AA-genotype. However, as already stated above, sample numbers in the Ghent subsample were small, and this observation could be due to chance. A statistical analysis stratifying for audiogram shape but using higher sample numbers might find an explanation for the aberrant picture observed in the Ghent subsample.

Previously, other ARHI susceptibility genes, such as *KCNQ4*, *NAT2* and others, have been proposed (30–33). Interestingly, *KCNQ4* was one of the candidate genes in this study. Despite the fact that the samples in the current study were collected identically to the samples for the second population used in the Van Eyken *et al*. (32) study, we failed to replicate our previous findings. The highest rank a *KCNQ4* SNP obtained within the analysis of all samples combined was 71. *NAT2*, *GSTM1*, *GSTT1* and *APOE* were not among the candidate genes selected for the current study.

GRHL2 is a transcription factor that is widely expressed in a variety of epithelial tissues (34). In the inner ear, *GHRL2* is

Table 7. *GRHL2* fine mapping (statistical analysis of all samples combined)

SNP number	SNP name	Coordinate	MAF	Homogeneity	*P*-value	OR
18	rs10955255	102605581	0.411	0.2948	**8.38×10^{-5}**	1.27
20	rs2127034	102611574	0.484	0.5885	**0.000160**	0.80
21	rs13263539	102613455	0.484	0.5854	**0.000249**	0.80
22	rs606338	102621097	0.192	0.3180	**0.026085**	1.19
23	rs1981361	102621953	0.412	0.0728	**0.000529**	0.81
25	rs17398001	102623393	0.163	0.6165	**0.007620**	0.80
27	rs3735709[a]	102624650	0.026[b]	0.3023	0.841249	0.97
52	rs17399841	102685908	0.052	0.4230	**0.034905**	1.34
53	rs632216	102686966	0.035[b]	0.9767	**0.041068**	0.71
55	rs548187	102696425	0.003[b]	0.5834	**0.033314**	0.32
63	rs7827945	102732501	0.255	0.1364	**0.026129**	0.86

Significant *P*-values have been indicated in bold.
Only significant SNPs are shown in addition to the non-synonymous SNP rs3735709 in exon 2. For an overview of all 67 SNPs, see Supplementary Material, Table S2.
[a]The NCBI information for rs3735709 is erroneous. According to NCBI the A-allele of the reference sequence is substituted for a T-allele, thereby changing lysine into isoleucine at amino acid position 9. However, by sequencing exon 2 in 32 individuals we consistently found the A-allele to be substituted for a G-allele, leading to an arginine at amino acid position 9.
[b]Results for SNPs with MAF <0.05 may not be reliable. These SNPs were also omitted from Figure 2.

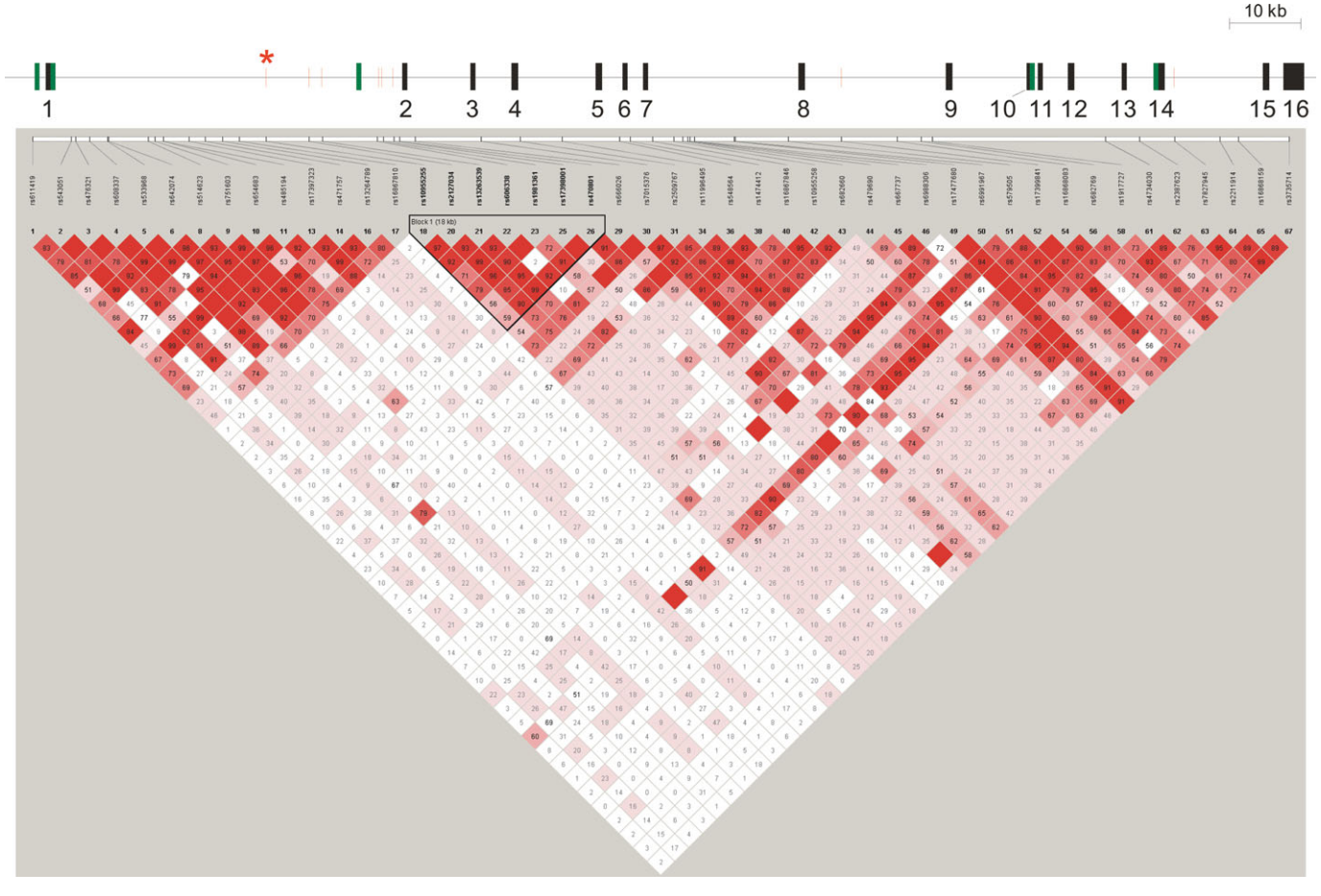

Figure 2. Genomic organization and LD within *GRHL2*. LD patterns were generated by Haploview. D′ is depicted. SNPs with MAF <0.05 were omitted from the LD analysis. The 18 kb LD-block that was manually imposed in Haploview to determine the risk haplotype is indicated. In the upper part of the figure the genomic organization of *GRHL2* is depicted. Exons are indicated with black boxes, putative promoter regions as predicted by Eldorado (Genomatix) are indicated with green boxes, and significantly associated SNPs are indicated with red lines. The most significantly associated SNP, rs10955255, is indicated with a red asterisk.

Table 8. Determination of the risk haplotype in the associated 18 kb LD block

Haplotype[a]	Frequency	*P*-value
GCAATAGAG	0.505	**3.0×10^{-4}**[b]
ACGGAGGAA	0.099	0.3690
ACGGTGGAG	0.097	0.4903
ACGGTGGCG	0.089	**0.0305**
ACGGTAGAG	0.042	0.0882
GCGGAGGAA	0.030	0.9027
ACGGAGGCG	0.026	**0.0122**
GCGGTAGCG	0.021	0.3585
ATGGAGGAA	0.017	0.9812
GCGGTGGCG	0.011	0.9933

Significant *P*-values have been indicated in bold.
[a]Haplotypes were deduced for the following SNPs: rs10955255, rs812890, rs2127034, rs13263539, rs606338, rs1981361, rs12114698, rs17398001 and rs470801. Rs812890 and rs12114698 were omitted from Figure 2 because they had a MAF of <0.05.
[b]Permutated *P*-value is 0.0005 (10 000 permutations).

expressed in cells lining the cochlear duct, most prominent during embryonic development and less during early postnatal stages (35). *GHRL2* belongs to a family of homologues of the *Drosophila* gene g*rainyhead* (34,36). In *Drosophila*, g*rainyhead* is critical for the regulation of several important developmental processes and homozygous mutations in this gene are embryonic lethal (34). The three mammalian homologues (*GRHL1* to *3*) are expected to be of equal importance during mammalian embryogenesis as all three genes are highly expressed in the developing epidermis. Interestingly, they display subtle differences in timing and level of expression, indicating that the three genes may not be redundant (37).

A frameshift mutation in *GHRL2* (1609–1610insC), leading to a truncation in exon 14 if the mRNA would be translated into protein, is responsible for DFNA28 autosomal dominant hearing loss (35). So far, no additional mutations in *GRHL2* have been described. The hearing loss in the family segregating c.1609–1610insC can be categorized as mild to moderate across all frequencies in the initial stages, but progressing towards severe hearing loss of the high frequencies in the fifth decade. The age at onset is variable, with the youngest patient diagnosed in his first decade. As such, DFNA28 hearing loss does not completely match the typical features observed in ARHI, but some properties certainly correspond, such as the progressive and sensorineural nature, and the fact that the high frequencies are most affected later in life. Although additional transcription factors have been implicated in autosomal dominant types of hearing loss before [EYA4 (eyes absent, drosophila, homologue of, 4) – DFNA10 (38) and POU4F3 (pou domain, class 4, transcription factor 3) – DFNA15 (39)], it was rather unexpected that a transcription factor with an important function during developmental stages was identified as the gene responsible for childhood-onset progressive hearing loss. However, this fact probably implies that, besides functioning during embryonic development, *GRHL2* also functions in epithelial cell maintenance throughout life (35). An impaired maintenance, albeit to a lesser degree, is most probably also the underlying pathological reason of *GRHL2*s involvement in a form of late-onset hearing impairment such as ARHI. Being aware of more recent clinical data, especially for DFNA15 (40), it is not unlikely to find that a transcription factor is involved in ARHI. Moreover, another transcription factor has been implicated recently in late-onset disease; *TCF7L2* (transcription factor 7-like 2) was established as susceptibility gene for type 2 diabetes (41,42). Therefore, although transcription factors do not pose obvious candidates for late-onset diseases, the involvement of *GRHL2* in ARHI is not exceptional at all.

As we excluded a non-synonymous SNP from exon 2 as putative causative SNP, and as exon 2 is not actually part of the 18 kb LD-block containing the most significant SNPs, it is most likely that the causative variant(s) will reside in the 18 kb LD-block located in intron 1. Interestingly, this intronic region contains a few evolutionary conserved regions, one of which coincides with a predicted promoter region. GRHL2 has at least two isoforms (Uniprot, URL:http://www.ebi.uniprot.org/entry/Q6ISB3). The complete protein (isoform 1) consists of 625 amino acids. Isoform 2 is encoded by an mRNA with an alternatively spliced exon 1 that is extended with 60 nucleotides from intron 1, immediately downstream of exon 1. A stop codon is present in this alternative 3′ end of exon 1 which may force the translation start of isoform 2 towards a methionine in exon 2. This would lead to an isoform 2 that lacks the first 16 amino acids of isoform 1. The first three exons of *GRHL2* encode a transcriptional activation domain (34). Isoform 2 may therefore regulate transcription differently compared to isoform 1. It is tempting to speculate that isoform 2 might be driven both by a general *GRHL2* promoter upstream of exon 1 and by an alternative promoter that is localized in intron 1. Alternatively, the expression of yet another, so far unidentified isoform may be driven by the alternative promoter in intron 1. A common variant present in this alternative promoter may be responsible for either increased or decreased expression levels in subjects who are susceptible to ARHI. We did not succeed in amplifying *GRHL2* with RT–PCR on lymphocyte mRNA. Indeed, the GNF SymAtlas (Genomics Institute of the Novartis Research Foundation, URL:http://symatlas.gnf.org/SymAtlas/) indicates that expression levels in lymphocytes are low. This implies that testing for an association between *GRHL2* expression levels and ARHI cannot easily be achieved and no evidence for our hypothesis can be provided in this way. To further address the identification of the causative variants, the 18 kb region of intron 1 can be resequenced in risk haplotype carriers and non-carriers, in order to document all common variation that is present in this region. Subsequent association studies should provide several putative causative variants, whose involvement in ARHI will be studied further by means of relevant functional studies.

It is well established that transcription factors obtain functional diversity through the presence of tissue-specific isoforms and through the formation of homo- and heteromeric complexes (34,36). Alternative splicing can generate specific isoforms and as such modulate DNA binding specificity or affinity, produce activators and repressors from the same gene and modulate dimerization (43). GRHL2 is known to homodimerize and to form heterodimers with GRHL1 (34) and GRHL3 (36). However, elucidation as to which GRHL2 isoforms and homo- and/or heterodimers are present in the inner ear is necessary. The functional properties of GRHL2

within the inner ear will also critically depend on the presence of its target genes. Human homologues of *Drosophila grainyhead* target genes, like *EN-1* (human engrailed homologue) have been identified, but it remains to be seen which of these genes are regulated by GRHL2 as most of the efforts have concentrated on the identification of target genes for GRHL1 (34). So far no mouse model is available, although a mouse lacking *Grhl2* may be imminent (37). However, for our purposes, i.e. the study of a maintenance role for GRHL2 in cochlear epithelial cells, a conditional knockout in which the gene can be switched off in adult inner ear, would be a more relevant animal model.

In conclusion, our candidate gene approach revealed *GRHL2* as ARHI susceptibility gene, as highly significant associations were observed for the complete sample set and replication was found in two independent populations. Fine mapping localized the region of interest in an 18 kb LD-block within intron 1, but further experiments will be necessary to identify the causative variant(s).

MATERIALS AND METHODS

Subjects

The subjects have previously been described (29,31). Briefly, Caucasian volunteers, 53–67 years of age, from nine centers in seven European countries, were collected via population registries or via audiological consultations. If the subjects were collected via hearing health services, the subjects' spouses were also included. All subjects underwent an otoscopic investigation. Subjects with ear diseases potentially affecting hearing thresholds were excluded from the study. All subjects completed an extended questionnaire detailing medical history and exposure to environmental factors. In general, subjects with pathologies that could potentially influence their hearing thresholds were excluded according to an extensive exclusion list (32). Air conduction thresholds were measured at 0.125, 0.25, 0.5, 1, 2, 3, 4, 6 and 8 kHz, and bone conduction at 0.5, 1, 2 and 4 kHz for all participating volunteers. Audiological exclusion criteria were a conductive gap of more than 15 dB averaged over 0.5, 1 and 2 kHz in one or both ears or asymmetrical hearing impairment with a difference in air conduction thresholds exceeding 20 dB in at least two frequencies between 0.5, 1 and 2 kHz. Informed consent was obtained from all volunteers. The study was approved by the ethical committees of the respective recruiting centers.

Z-score calculations and sample selection

Z-scores were calculated as described previously (32,44). Briefly, frequency-specific thresholds were converted to sex and age independent *Z*-scores based on the ISO 7029 standard (45). These *Z*-scores represent the number of standard deviations the actual hearing threshold differs from the median at a given frequency. Subjects whose hearing is better than the age and sex specific median at a certain frequency have a negative *Z*-score. As a measure of high frequency hearing impairment, the *Z*-scores at 2, 4 and 8 kHz were averaged (Z_{high}). This was done separately for both ears but only the *Z*-score for the better hearing ear was used. After excluding phenotypic outliers for Z_{high}, we selected the 34% best and 34% worst hearing subjects within each center, for males and females separately (Table 1 and Supplementary Material, Fig. S1). Subsequently, the phenotype was dichotomized by labeling the two distribution extremes as cases and controls.

Candidate gene and SNP selection

A list of candidate genes was generated based on the literature and information available on several websites, including the Hereditary Hearing Loss website (URL: http://webh01.ua.ac.be/hhh/), the Jackson Laboratories Hereditary Hearing Impairment in Mice website (URL: http://www.jax.org/hmr/) and the Sanger Institute Deaf Mouse Mutants website (URL: http://www.sanger.ac.uk/PostGenomics/mousemutants/deaf/). The resulting list of 70 candidate genes mainly consisted of monogenic hearing loss genes identified in mice and men in addition to several strong functional candidate genes (Table 2 and Supplementary Material, Table S1). Subsequently, SNPs were selected in all candidate genes. To achieve this, a list of all SNPs that were present in the HAPMAP database (release no. 16) (URL: http://www.hapmap.org/) in the genomic region of the candidate gene and in a region 3000 bp upstream of the gene (putatively containing regulatory elements) was used as an input file for the MARKER computer program (URL: http://www.gmap.net/marker). Based on the HAPMAP data for these SNPs, MARKER was used to select the tag SNPs. In addition, SNPs with proven or putative biological relevance were selected from dbSNP (URL: http://www.ncbi.nlm.nih.gov), the SNPeffect database (URL: http://snpeffect.vib.be), the Genetic Association database (URL: http://geneticassociationdb.nih.gov), and the ABI SNP database (URL: http://appliedbiosystems.com). All selected SNPs had a MAF of 0.05 or higher. The exact number of selected SNPs per gene is shown in Supplementary Material, Table S1. The designability of each selected SNP for the Illumina GoldenGate genotyping assay was checked in consultation with Illumina (San Diego, CA, USA). SNPs, which were undesignable (score of 0) or which had a low designability score (score of 0.5) were replaced by other, if possible, equally informative SNPs, depending on the output of a Tagger analysis (URL: www.broad.mit.edu/mpg/tagger). All 768 SNPs on the final SNP list had the maximum designability score of 1.0. A classification of the selected SNPs is given in Table 3.

SNP genotyping

Assay design and genotyping of all SNPs on all selected samples were performed by Illumina (San Diego). For each center, four samples were genotyped in duplicate. As a control for Illumina's genotyping accuracy, a selection of SNPs was genotyped in our laboratory on a subset of samples using either the AcycloPrime-Fluorescence Polarization (FP) SNP Detection System (PerkinElmer Life Sciences, Boston, MA, USA) or the SNaPshot™ Detection Method (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions and as described previously (32). In case of discrepancies, dye terminator cycle sequencing

was performed using ABI procedures and equipment (Applied Biosystems). The sequencing result was considered the gold standard.

GRHL2 fine mapping

For *GRHL2* fine mapping purposes, we selected additional tag SNPs in the genomic region of the gene and 3000 bp upstream, according to the information that was present in HAPMAP release no. 22. There was no cut-off for the MAF. The relevant HAPMAP data were dumped into Haploview and tag SNPs were selected using the default settings ($r^2 = 0.8$; aggressive tagging) of Tagger. This resulted in a total of 44 additional tag SNPs, excluding those SNPs that had previously been genotyped by Illumina ($n = 26$). In addition to the selected tag SNPs, a non-synonymous SNP located in exon 2 (rs3735709) was added to the list, resulting in a total number of 45 SNPs that were genotyped by Kbioscience (Hoddesdon, UK).

Additional bioinformatical analysis

LD patterns of candidate genes were determined with the default settings of Haploview 3.32 (URL: www.broad.mit.edu/mpg/haploview/) using all available genotyping data. Haploview 3.32 was also used to determine the risk haplotype after manually imposing the LD block of interest. Prediction of promoter and regulatory regions in *GRHL2* was performed with the Eldorado software package (Genomatix; URL: http://www.genomatix.de/). Comparative analysis of human and mouse *GRHL2* genomic sequences was performed using VISTA Tools (URL: http://genome.lbl.gov/vista/) (46,47).

Data polishing

The first stage in the data polishing process consisted of removing all samples with 10% or more missing genotypes. Subsequently, SNP assays with more than 4% missing genotypes among all genotyped subjects were excluded. Gender mis-specification was checked by matching the gender deduced from sex-specific control loci with the gender that was provided in the sample list. A subsequent step consisted of Hardy–Weinberg equilibrium testing for every approved SNP on all approved samples by χ^2-testing. Based on a significance level of 0.001 all SNP assays were in Hardy–Weinberg equilibrium. The final step of the polishing process consisted of the detection and removal of genetic outliers, using CHECKHET (48) and GRR (URL: http://bioinformatics.well.ox.ac.uk/GRR) (49). As a homogeneous genetic background of each independent sample set enhances the power of a genetic association study, CHECKHET can be used to detect small numbers of subjects with a different genetic background when compared to the genetic background of the majority of the tested samples. The presence of related individuals in association studies using unrelated samples could lead to incorrect conclusions. GRR detects putative relatives on the basis of the fact that related individuals share an excess of alleles identical by state. In addition, GRR is also capable of detecting sample duplications. The cut-off value for exclusion was 1.75 on a scale of 2.0 identical by state.

Statistical analysis

Logistic regression was performed on the genotyping data of samples from the nine centers as a whole and on the data of samples from the nine centers separately. As the presence of population stratification, i.e. the existence of (often unobserved) subgroups within the study population that differ both in allele frequencies and in baseline risk of disease, may lead to spurious associations, the subject origin was taken into account in the combined analysis. We assumed an additive genetic effect on the logit scale so that genotypes were coded as 0, 1 or 2 and that genotype was treated as a continuous covariate in the logistic regression model. To test for homogeneity across the nine recruiting centers in the combined analysis, we first fitted a full model containing main effects for center (eight dummy variables) and genotype, and interaction terms between center and genotype. Using a likelihood ratio test, this model was compared with a reduced model without the eight interaction terms. When this test was non-significant, a homogeneous effect of the genotype across all centers was assumed. The model was refitted and the genotype effect was tested using a likelihood ratio test. The main effects of center were retained in this model to account for small artificial differences in 'disease prevalence' because of lost samples due to poor DNA quality or 'no genotype' calls. We realize that additional hidden population structure might be present within populations, but the impact of this on the results of genetic association studies has been demonstrated to be of minor importance within Europe (50). ORs were calculated by exponentiating regression coefficients obtained from the logistic regression model. The FDR was calculated according to Storey and Tibshirani (51).

An allelic heterogeneity test was performed for the *GRHL2* region in order to test how many association signals were present (52). Briefly, we started with a logistic regression model containing only the origin and the genotype of the most significant SNP (rs10955255). Unlike the initial significance test, a genotypic model (two degrees of freedom) was fitted for this latter SNP, making no assumption about the mode of inheritance. Subsequently, we added each of the remaining significant SNPs separately to the model and tested if the model fit significantly improved (likelihood ratio test, 1 degree of freedom, additive model). In case the added SNP gave no significant improvement in model fit, we concluded that the added SNP represented the same association signal as the most significant SNP, and that its significance was merely due to LD with the most significant SNP. If adding a SNP significantly improved the model fit, we concluded that this particular SNP represented an independent association signal.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

The authors would like to express their most sincere gratitude to all the volunteers who have participated in this study.

Conflict of Interest statement. None declared.

FUNDING

This work is supported by grants from the European Community (5th Framework project QLRT-2001-00331), the British Royal National Institute for Deaf and hard of hearing individuals (RNID), the Flemish organization for scientific research (FWO grant G.0131.04) and the University of Antwerp (TOP project).

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