Resequencing of the auxiliary GABAB receptor subunit gene KCTD12 in chronic tinnitus

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INTRODUCTION

Tinnitus is a common and often incapacitating hearing disorder marked by the perception of phantom sounds. Susceptibility factors remain largely unknown but GABAB receptor signaling has long been implicated in the response to treatment and, putatively, in the etiology of the disorder. We hypothesized that variation in KCTD12, the gene encoding an auxiliary subunit of GABAB receptors, could help to predict the risk of developing tinnitus. Ninety-five Caucasian outpatients with a diagnosis of chronic tinnitus were systematically screened for mutations in the KCTD12 open reading frame and the adjacent 3’ untranslated region by Sanger sequencing. Allele frequencies were determined for 14 known variants of which three (rs73237446, rs34544607, and rs41287030) were polymorphic. When allele frequencies were compared to data from a large reference population of European ancestry, rs34544607 was associated with tinnitus (p = 0.04). However, KCTD12 genotype did not predict tinnitus severity (p = 0.52) and the association with rs34544607 was weakened after screening 50 additional cases (p = 0.07). Pending replication in a larger cohort, KCTD12 may act as a risk modifier in chronic tinnitus. Issues that are yet to be addressed include the effects of neighboring variants, e.g., in the KCTD12 gene regulatory region, plus interactions with variants of GABA_B1 and GABA_B2.

Keywords: KCTD12, association analysis, tinnitus, cortical inhibition

MATERIALS AND METHODS

In 95 German outpatients (67 men and 28 women, age 50.6 ± 12.1 years, mean ± SD) consulting for chronic tinnitus, the diagnosis was confirmed by a detailed neurootological examination...
including otoscopy, stapedius reflexes, middle ear pressure measurements, and pure tone audiometry. For the present study, only patients with subjective tinnitus were included. Tinnitus severity was assessed by the Tinnitus Questionnaire (TQ) (Goebel and Hiller, 1994). An additional 50 subjects with chronic tinnitus (40 men and 10 women, age 49.3 ± 11.3 years, mean ± SD) formed an extension sample and underwent the same diagnostic workup as outlined above. All participants were Caucasians and a majority originated from the Upper Palatinate region of Bavaria. All provided informed consent and the study was approved by the local ethics committee at the University of Regensburg.

Genomic DNA was extracted from lymphocytes using standard procedures prior to amplification of the KCTD12 open reading frame and adjacent 3' sequence by PCR. Briefly, two overlapping amplicons of 438 bp (a) and 819 bp (b) were generated using the following primer pairs: 5'-CGG TTG CAG CTC CTG AGT-3' (forward, a), 5'-AGC TCT GCC AGC TCG AAG TA-3' (reverse, a), 5'-CTC GTG CCC GAC TAC TT-3' (forward, b) and 5'-GAC AGG TTG CAG AC-3' (reverse, b). PCR products were purified with ExoSAP-IT (GE Healthcare, Freiburg, Germany) for Sanger sequencing, and for the identification of variants against the human genome reference (Genome Reference Consortium Build 37, February 2009 release). In the extension sample, only amplicon b was sequenced. Multiple sequence alignments were conducted with computational annotations of the next (Siepel et al., 2005). The level of statistical significance was set at p < 0.05. All p-values are uncorrected for multiple testing.

For estimating the functionality of confirmed sequence variants, evolutionary conservation in primates was assessed with a phylogenetic hidden Markov model-based method, phastCons, that describes the process of DNA substitution at each site in a genome and the way this process changes from one site to the next (Siepel et al., 2005). Computational annotations of SNP function (Xu and Taylor, 2009) were obtained from the SNPinfo WebServer (URL: http://snpinfo.niehs.nih.gov/snpfunc.htm, accessed Dec. 2011). In silico predictions of structural effects at the amino acid level were based on information from homologous proteins using metaPrDOS at default parameters (Ishida and Kinoshita, 2008).

RESULTS

We confirmed the existence of two coding variants, F87F (rs73237446) and T178T (rs34544607), plus one previously described, non-coding variant in the gene’s 3' UTR (rs41287030) at heterozygosities of 0.01, 0.10, and 0.02, respectively. All genotype distributions conformed to the Hardy–WeinbergTABLE 1 | Allele frequencies for the KCTD12 sequence screened in subjects with chronic tinnitus as compared to frequencies in a large control population.

| dbsNP ID    | chr13 position | Major>minor allelesa | Variant amino acid | MAF in chronic tinnitus (2N)b | MAF in controls (2N)c | p  |
|-------------|----------------|---------------------|-------------------|-------------------------------|----------------------|----|
| rs141180437 | 77460,118      | C>T                 | P56S              | 0.0000 (190)                  | 0.0000 (6972)        | n.s.|
| rs116710456 | 77460,080      | G>A                 | Q68Q              | 0.0000 (190)                  | 0.0003 (6858)        | n.s.|
| rs14301358  | 77460,078      | C>T                 | P96L              | 0.0000 (190)                  | 0.0000 (6844)        |     |
| rs694997    | 77460,068      | G>A                 | L72L              | 0.0000 (190)                  | 0.0003 (6872)        | n.s.|
| rs146434030 | 77460,065      | C>A                 | A73A              | 0.0000 (190)                  | 0.0000 (6894)        | n.s.|
| rs73237446  | 77460,023      | C>T                 | F87F              | 0.0053 (190)                  | 0.0089 (6890)        | n.s.|
| rs141477426 | 77460,015      | G>A                 | R90H              | 0.0000 (190)                  | 0.0001 (6858)        | n.s.|
| rs144225285 | 77459,981      | C>T                 | L101L             | 0.0000 (190)                  | 0.0000 (6756)        | n.s.|
| rs34544607  | 77459,750      | G>C                 | T178T             | 0.0458 (262)                  | 0.0263 (5058)        | 0.07|
| rs139291676 | 77459,507      | C>T                 | P259P             | 0.0000 (262)                  | 0.0000 (7018)        | n.s.|
| rs151278314 | 77459,394      | C>T                 | T297M             | 0.0000 (262)                  | 0.0000 (7020)        | n.s.|
| rs142368706 | 77459,383      | G>A                 | A301T             | 0.0000 (262)                  | 0.0000 (7020)        | n.s.|
| rs140689403 | 77459,359      | A>G                 | S309G             | 0.0000 (262)                  | 0.0001 (7020)        | n.s.|
| rs41287030  | 77459,221      | C>T                 | –                 | 0.0076 (262)                  | –                    | –   |

*Fisher’s exact tests were used to assess allelic association.
*aNucleotides on the transcribed strand.
*bCall rates of 85% were achieved in the first round of screening amplicon b.
*cReference population of European ancestry from the NHLBI GO Exome Sequencing Project. Data retrieved with the Exome Variant Server (URL: http://evs.gs.washington.edu/EVS/), accessed December 2011.

MAF, minor allele frequency.
equilibrium ($p > 0.75$). No novel sequence variants emerged and 11 $KCTD12$ variants listed in dbSNP were absent from our sample (rs141180437, rs116710456, rs143013358, rs694997, rs146434030, rs141477426, rs144225285, rs139291676, rs151278314, rs142368706, and rs140689403, Table 1). When allele frequencies in subjects with chronic tinnitus were compared to reference frequencies from a large control population of European ancestry, an increased prevalence of the minor allele was noted for T178T ($0.0494$ vs. $0.0263$, $p = 0.04$). To put this finding into perspective, the original screening sample was augmented by 100 chromosomes from a second set of patients, whereupon the MAF in cases dropped to $0.0458$ for rs34544607, weakening the association with tinnitus ($p = 0.07$). Power simulations, based on the entire sample of patients diagnosed with chronic tinnitus and on ESP control data, indicated that we should expect a statistical power of $>80\%$ to detect a susceptibility factor with an allelic relative risk of $>1.77$ for the T178T variant. The number of tinnitus cases needed to reach this power was estimated at 363.

We next examined whether $KCTD12$ variants could serve as predictors of tinnitus severity. Overall, TQ scores followed a Gaussian distribution (Figure 1) and averaged $37.1 \pm 16.3$ (mean $\pm$ SD) out of 84 points ($N = 144$). By this measure, tinnitus was rated mild ($0$–$30$ points) in $55$ subjects ($38.2\%$), moderate ($31$–$46$ points) in $46$ subjects ($31.9\%$), severe ($47$–$59$ points) in $29$ subjects ($20.1\%$), and extreme ($60$–$84$ points) in $14$ subjects ($9.7\%$). There was no significant difference in mean TQ scores or in the degree of concomitant hearing loss between carriers and non-carriers of the minor allele at rs34544607 ($p = 0.52$ and $p = 0.48$, respectively, $t$-test, Figure 2). A positive family history of tinnitus in first-degree relatives did not predict rs34544607 genotype ($p = 0.67$, Fisher’s exact test). As we encountered only one carrier of rs73237446, and only two carriers of rs41287030, the interplay of these substitutions with tinnitus severity, hearing loss, or with a family history of tinnitus could not be fully judged.

Using the degree of evolutionary conservation as a surrogate parameter of functionality, both rs73237446 and rs34544607 scored high on the comparative genomics scale (Figure 3). Further in silico analyses confirmed that rs73237446 maps to the potassium channel tetramerization domain (Figure 3) whereas residue 178, encoded by rs34544607, maps to a disordered region of $KCTD12$ (Figure 4) which may affect the molecular recognition of proteins and DNA. The non-coding variant rs41287030 is only poorly conserved among primates but could have acquired a functional role in the recent past. Thus, rs41287030 would appear to alter a micro RNA binding site and may thereby inhibit protein translation (see the corresponding SNPinfo entry for prediction results).

**DISCUSSION**

Screening of the $KCTD12$ ORF in chronic tinnitus extends preliminary results on genomic variation as obtained from 88 subjects.
with congenital deafness (Kuo, 2005). As in the earlier study, no novel sequence variants were identified. However, a trend was observed for association of chronic tinnitus with a highly conserved, synonymous substitution, rs34544607. The relevance of this finding is unclear in view of the moderately sized sample and the use of an external reference population. It is conceivable that some control subjects from the ESP may have experienced mild, subclinical forms of tinnitus, increasing the likelihood of a type II error. Pending replication of this association trend at a larger scale, the mechanism by which rs34544607 can affect hearing also remains to be elucidated. Possible explanations for synonymous mutations’ functionality are offered by interference with RNA processing, or by changes in translation kinetics that affect protein folding (Sauna and Kimchi-Sarfaty, 2011). Phenotypically, rs34544607 carriers may be indistinguishable from other subjects unless treated with baclofen or another GABAB receptor agonist. If rs34544607 truly impacts on GABAB signaling, we should expect electrophysiological measures of cortical inhibition to discriminate between carriers and non-carriers. Electrophysiological data (motor threshold, short-interval intracortical inhibition, intracortical facilitation, and cortical silent period) were available only in a subset of our sample and did not suggest a major effect. The degree of hearing loss did not predict rs34544607 carrier status but further stratification by etiology (noise-induced vs. congenital) is recommended in future studies. With regard to rs41287030, the current lack of publicly available control data and a MAF < 0.01 in tinnitus subjects call for a re-examination in a larger population of affecteds and controls in order to test for a possible association with the phenotype.

Taken together, the present results implicate genetic variation in a GABA_B receptor auxiliary subunit as a possible risk modifier in chronic tinnitus. More research is also invited to address KCTD12 promoter variants, and to explore the interaction with variants in genes encoding other elements of the receptor complex, e.g., GABA_B1 and GABA_B2 proteins.

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