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Clinical and Genome-Wide Analysis of Cisplatin-Induced Tinnitus Implicates Novel Ototoxic Mechanisms

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Abstract

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Disclosure of Potential Conflicts of Interest

The authors declare no potential conflicts of interest.

Purpose: Cisplatin, a commonly used chemotherapeutic, results in tinnitus, the phantom perception of sound. Our purpose was to identify the clinical and genetic determinants of tinnitus among testicular cancer survivors (TCS) following cisplatin-based chemotherapy.

Experimental Design: TCS (n= 762) were dichotomized to cases (moderate/severe tinnitus; n=154) and controls (none; n=608). Logistic regression was used to evaluate associations with comorbidities and SNP dosages in GWAS following quality control and imputation (covariates: age, noise exposure, cisplatin dose, genetic principal components). Pathway over-representation tests and functional studies in mouse auditory cells were performed.

Results: Cisplatin-induced tinnitus (CisIT) significantly associated with age at diagnosis (P=0.007) and cumulative cisplatin dose (P=0.007). CisIT prevalence was not significantly greater in 400 mg/m^2-treated TCS compared to 300 (P=0.41), but doses >400 mg/m^2 (median 580, range 402–828) increased risk by 2.61-fold (P<0.0001). CisIT cases had worse hearing at each frequency (0.25–12 kHz, P<0.0001), and reported more vertigo (OR=6.47; P<0.0001) and problems hearing in a crowd (OR=8.22; P<0.0001) than controls. Cases reported poorer health (P=0.0005) and greater psychotropic medication use (OR=2.4; P=0.003). GWAS suggested a variant near *OTOS* (rs7606353, P=2×10^{-6}) and *OTOS* eQTLs were significantly enriched independently of that SNP (P=0.018). OTOS overexpression in HEI-OC1, a mouse auditory cell line, resulted in resistance to cisplatin-induced cytotoxicity. Pathway analysis implicated potassium ion transport (q=0.007).

Conclusions: CisIT associated with several neuro-otological symptoms, increased use of psychotropic medication, and poorer health. *OTOS*, expressed in the cochlear lateral wall, was implicated as protective. Future studies should investigate otoprotective targets in supporting cochlear cells.

Terms:

cisplatin; hearing loss; tinnitus; ototoxicity; GWAS; adverse drug events; genetic architecture

INTRODUCTION

Approximately 9.6% of the U.S. population experiences tinnitus, the phantom perception of sound characterized by persistent ringing, buzzing, beeping, or hissing in the ears (1). Several studies have linked tinnitus to increased anxiety, depression, insomnia, and reduced productivity and quality of life (QoL) (2, 3). Nevertheless, the pathogenic mechanisms of this disorder remain poorly understood (4), and no pharmacological agents are approved to treat tinnitus (5); treatment is typically limited to cognitive behavioral therapy and management of associated anxiety (5, 6). Despite its etiological ambiguity, several risk factors have been shown to contribute to the development of tinnitus, including age, hearing loss, and the administration of ototoxic drugs (7, 8).

Cisplatin is one of the most commonly used chemotherapeutics, and is known for its potent ototoxic effects. It can lead to irreversible bilateral hearing loss in up to 80% of patients, with many experiencing permanent tinnitus (9–11). Although few studies have rigorously assessed tinnitus following cisplatin-based chemotherapy, approximately 40% of patients appear to experience at least some degree of tinnitus [6]. This is substantially higher than the rate observed in the general U.S. population, and in testicular cancer patients who did not

receive cisplatin-based chemotherapy (12%; [9]). Severe cases of tinnitus are also markedly increased in cisplatin-treated patients when compared to the general population (22% vs. 1–2%) (4) (9). One study of 1,409 testicular cancer survivors (TCS) noted that 19% of TCS treated with 100–400 mg/m^2 cisplatin exhibited symptoms of moderate/severe tinnitus, with that prevalence reaching 42% in the dose-intensive cisplatin chemotherapy group [9], suggesting a dose-dependent effect. It has been previously demonstrated that cancer survivors with neuropathy, hearing loss, and tinnitus were more likely to report poorer quality of life (QoL) than those with neuropathy alone (12). Another investigation confirmed worse perceived stress among cancer survivors with tinnitus (13).

Despite this documented effect on QoL, little is known about the risk factors and mechanisms of cisplatin-induced tinnitus (CisIT). The subjective nature of the disorder and lack of model systems hamper the elucidation of its pathophysiology. While genotype-phenotype associations promise to identify underlying molecular alterations, to our knowledge, no genome-wide association study (GWAS) of CisIT has yet been conducted. We previously characterized ototoxicity in our North American cohort of TCS receiving homogeneous cisplatin-based treatment. Among 488 TCS, 40% experienced some degree of tinnitus and 15.8% reported moderate/severe tinnitus. Further, tinnitus was significantly correlated with reduced hearing at each frequency examined ($P < 0.001$) (9). In this study, we comprehensively evaluate the role of clinical characteristics and modifiable risk factors on the development of tinnitus after cisplatin-based chemotherapy in a cohort of 762 TCS, and identify genetic determinants of CisIT through GWAS. In addition, we conduct pathway analyses and in vitro experiments to validate and contextualize our results.

METHODS

Patients and Study Design

All patients were enrolled in the Platinum Study, which includes eight cancer centers in the United States and Canada. Eligibility criteria included: men diagnosed with histologically or serologically confirmed germ cell tumor (GCT), age <55 years at diagnosis and >18 years at enrollment, treatment with first-line cisplatin-based chemotherapy regimen for either initial GCT or recurrence after active surveillance, with no antecedent chemotherapy for another primary cancer, no salvage chemotherapy, and routine follow-up at the participating site. All patients were disease-free at the time of clinical evaluation and provided written consent for study participation, access to medical records, and genotyping. Study procedures were approved by the Human Subjects Review Board at each institution. The studies were conducted in accordance with the U.S. Common Rule.

Assessments

Patient data were either ascertained at clinical follow-up or abstracted from medical records according to a standardized protocol (Figure S1). Data abstracted from medical charts included age at GCT diagnosis, GCT tumor characteristics, treatment regimen with cumulative dose and number of cycles, and BMI at the initiation of treatment. Data collected at clinical evaluation included age and BMI, audiometric studies, self-reported questionnaires, and genotyping. Pure-tone air conduction thresholds were measured

bilaterally on a subset of 602 survivors at the frequency range 0.25–12 kHz to quantify hearing. The geometric mean of hearing thresholds across cisplatin-affected frequencies (4–12 kHz) was used to quantify cisplatin-induced hearing loss as previously described (9). Self-reported ototoxicity and neurotoxicity were assessed with two validated questionnaires: the EORTC-Chemotherapy Induced Peripheral Neuropathy 20 item questionnaire (CIPN20) and the Scale for Chemotherapy-Induced Neurotoxicity (SCIN)(14).

Hypertension, hypercholesterolemia, use of psychotropic medication (antidepressants, anxiolytics, and antipsychotics), overall health, tobacco use, alcohol consumption, and noise exposure were also ascertained at follow-up as patient-reported outcomes. Hypertensive subjects indicated receiving a diagnosis of high blood pressure and taking prescription medication for it at evaluation. Hypercholesterolemia subjects answered “yes, current” to the question: “Have you ever taken prescription medication for high cholesterol?” The use of psychotropic medication was assessed as in Fung et al. (15), and was based on whether a patient had been taking psychotropic medication for at least one month at the time of clinical evaluation. Self-reported health was rated on a poor-excellent scale in response to the question “How would you rate your health?” Values were numerically converted for association testing (poor/fair = 1, good = 2, very good = 3, excellent = 4). Exposure to noise was assessed using two questions: “Have you ever had a job where you were exposed to loud noise for 5 or more hours a week? (Loud noise means noise so loud that you had to speak in a raised voice to be heard)” and “Outside of a job, have you ever been exposed to steady loud noise or music for 5 or more hours a week? (Examples are noise from power tools, lawn mowers, farm machinery, cars, trucks, motorcycles, or loud music)”. After numeric conversion (Yes = 1, No = 0), overall noise exposure was computed as the sum of noise exposure in and outside of work. Alcohol consumption was assessed as the self-reported response to the question “During the past year, how many drinks of alcoholic beverage have you consumed on average? (1 drink = 12 oz. beer [1 can or bottle], 4 oz. glass of wine, 1 mixed drink or shot of liquor)” with the following options: Rarely/never (0), 1–3/month (1), 1/week (2), 2–4/week (3), 5–6/week (4), 1/day (5), 2–3/day (6), 4–5/day (7), and 6+/day (8). Tobacco use was assessed as the response to the following two questions: “Have you EVER smoked cigarettes?” with “Yes” (1) and “No” (0) options and “Do you CURRENTLY smoke cigarettes?”

Genotyping

Genotyping was done as previously described (16, 17). DNA was extracted from peripheral blood mononuclear cells. SNPs were genotyped in two phases at the RIKEN Center for integrative Medical Science (Yokohama, Japan). Set 1 was genotyped on the HumanOmniExpressExome-8v1-2_A chip (Illumina, San Diego, CA) and set 2 was genotyped on the InfiniumOmniExpressExome-8v1-3_A chip (Illumina, San Diego, CA). Samples were plated with inter- and intra-plate duplicates at random. Standard quality control (QC) was implemented in PLINK, and is outlined in Figure S2. Sample-level QC criteria included: sample call rate > 0.99, pairwise identity by descent < 0.125, coefficient of inbreeding F < 6 standard deviations from the mean, and genetically European as determined by principal component analysis (performed using SMARTPCA). SNP-level QC included: call rate > 0.99, and Hardy-Weinberg equilibrium (Chi-squared $P > 1 \times 10^{-6}$). QC was first

performed in each set independently and then the two sets were merged followed by additional QC. QC for batch effects was performed using a batch pseudo-GWAS where variants associated with batch pseudo-phenotype (10 genotypic PC-adjusted $P<10^{-4}$) were excluded. SNPs and samples passing QC criteria comprised the input set for imputation with EAGLE phasing, performed on the Michigan Imputation Server using the Haplotype Reference Consortium (18–20). SNPs with imputation $R^2 < 0.8$, MAF < 0.01, and INFO scores > 1.05 or < 0.3 were excluded. Only subjects who passed QC were included in the current study (Figure S2).

Case-Control Definition

Using SCIN (14), TCS were dichotomized to tinnitus cases/controls based on responses to the question: “Have you had in the last 4 weeks: Ringing or buzzing in the ears?” Cases responded “quite a bit/very much”, and controls responded “not at all”. Those answering “a little” were not included. We labeled “not at all”, “a little”, and “quite a bit/very much” as none, mild, and moderate/severe tinnitus. TCS were also asked: “Do you have: ringing and buzzing in the ears?” Tinnitus cases responding “no” to this question were excluded from analysis.

Genome-Wide Analyses

GWAS was done in PLINK v1.9 (21, 22) with logistic regression assuming additive effects, with cumulative cisplatin dose, age at diagnosis, self-reported noise exposure, and the first 5 genetic principal components (SMARTPCA (23)) as covariates. We performed imputation of missing covariates to avoid sample exclusion. Eight individuals with missing age at diagnosis were assigned an imputed age calculated as age at clinical follow-up minus median follow-up duration (5.2 years). Twelve individuals were missing values for cumulative cisplatin dose received, but each of these patients had information on the cycles of cisplatin received. Since one cycle of cisplatin is equivalent to 100 mg/m^2, these patients were assigned an imputed cumulative dose by multiplying the number of cycles they received by 100 (i.e. 3 cycles × 100 = 300 mg/m^2). No subjects were missing data on noise exposure. Genome-wide significance was set to $P<5 \times 10^{-8}$. A list of expression quantitative trait loci (eQTLs) was extracted from GTEx v7 using all 48 tissues with eQTL analysis (24). Conditional analysis was performed by adjusting for the lead single nucleotide polymorphism (SNP) as a covariate. SNP family enrichment was assessed using the empirical Brown’s combined p-value (25). Narrow-sense heritability was estimated with a genetic relationship matrix variance component model as implemented by GCTA with the same covariates as GWAS (26). Pathway over-representation analysis was performed using Gene Ontology (27, 28) pathways with the g:Profiler package(29) at a significance threshold of FDR q < 0.05 after attributing genes the p-value of the most significantly associated SNP in or within 25 kilobases of gene start/end sites (GENCODE GRCh37.p12).

Gene Expression and Toxicity in Cell Lines

OTOS expression data in different cancer cell lines was obtained from the Cancer Cell Line Encyclopedia (CCLE) (30). Drug sensitivity to cisplatin and other antineoplastic agents, measured as the area under the dose-response curve, was obtained from the Genomics of Drug Sensitivity in Cancer Project (31). Spearman correlation and linear regression was

performed between expression and sensitivity of cancer cell lines with non-missing *OTOS* expression in R 3.3.2.

Statistical Analysis

Data are presented with medians (ranges). Age was modeled continuously and in quartiles (ordinal). Cisplatin dose was treated continuously and in dose categories: <300, 300, 400, and >400 mg/m^2. Prevalence ratios (PR) were calculated to compare tinnitus prevalence by treatment or allele group, with normal approximation for 95% confidence intervals (CI) and χ^2 p-values reported. Logistic models were constructed to assess associations (odds ratios [OR]) with tinnitus in univariable and multivariable contexts. All tests were two-sided at a significance threshold of $P<0.05$. Results are presented as OR/PR (95% CI). All statistical analyses were performed in R 3.3.2 and plotted with ggplot2 (32) unless otherwise specified.

Cell Culture

HEI-OC1, a mouse auditory cell line derived from the inner ear organ of Corti, was cultured in complete media of DMEM (high glucose, L-glutamine and pyruvate) with 10% FBS (Corning Inc.; Tewksbury, MA). L929, a mouse fibroblast cell line, was cultured in complete media of EMEM with 10% FBS and 1% penicillin/ streptomycin. Cells were passaged 3-times/week, plated at 1×10^6 cells/T25 culture flask, and grown in a humidified chamber. HEI-OC1 was grown at 33°C with 10% CO_2 and L929 at 37°C with 5% CO_2. The authenticity of the cell lines was confirmed by IDEXX BioResearch (Columbia, MO) by measurement for interspecies contamination or misidentification, Case # 26495–2018 (HEI-OC1) and #32923–2018 (L929). Cell lines were also assessed for mycoplasma contamination using a MycoAlert Mycoplasma Detection Kit (Lonza; Rockland, ME) at the time of acquisition and prior to performing *in vitro* experiments.

Plasmid Preparation

OTOS protein vector, pOTOS (pPM-N-D-C-His; cat# PV401573) and blank control (pControl; PV001) vector were purchased from Applied Biological Materials, Inc. (Richmond, BC), with the blank control vector sequence being verified by the supplier. The plasmids were amplified into competent DH5α bacteria (Life Technologies Corporation; Grand Island, NY), expanded using 50μg/ml kanamycin selection, and isolated using PureYield Plasmid Midiprep (Promega Corporation; Madison, WI). Confirmation of *OTOS* cDNA sequence was performed at the University of Chicago DNA Sequencing Core.

Cell Viability Assay

After 24 hours of plating each cell line at 31,250 cells/cm^2 into 96-well plates, the cells were transfected in Opti-MEM (Life Technologies) with the addition of a complexed DNA mixture consisting of 1 part Lipofectamine 3000 (Fisher Scientific) with 3 μg pOTOS or pControl, as per manufactures' instructions. Transfection continued for 24 hours. Cisplatin (Sigma-Aldrich) was dissolved in a darkened hood using 0.9% saline. The solution was vortexed for 1 minute, sonicated for 10 minutes with no heat, and then filtered through a 0.2 μm syringe filter to obtain a 10 mM stock solution. Transfection media was exchanged for 5, 7.5, 10, 25 or 50 μM cisplatin in complete media or vehicle control. At 48 or 72 hours post-

drug treatment, 100 uL CellTiter-Glo (Promega Corporation) was added to each well, as per manufacturer's instructions. 135 μL of each well was transferred to a white Costar assay plate (Fisher Scientific) and luminescence was measured on a Synergy-HT plate reader (BioTek Corporation; Broadview, IL). Four independent experiments were performed and within an experiment, each concentration was evaluated in triplicate. IC_{50} values were calculated through non-linear regression of the log cisplatin concentration vs. normalized response curve using Prism6 software.

Quantitative PCR.

Cells were plated at 31,250 cells/cm^2 into 12-well plates for qPCR collections. After 24, 48, 72, and 96 hours of transfection (corresponding to 0, 24, 48, and 72 hours after cisplatin treatment), cells were pelleted following a brief trypsinization and stored at −80°C. RNA was isolated using the RNeasy Plus Kit (Qiagen; Germantown, MD) followed by reverse transcription of 200ng RNA into cDNA with the High Capacity cDNA reverse transcription kit (Fisher Scientific). Mouse *OTOS* (Mm01292235_g1) expression was tested in HEI-OC1 and L929 cells, and was found to be minimally expressed. Expression of human *OTOS* was determined with qPCR using Taqman primer *OTOS* (Hs00964785_m1) and compared to mouse Actβ (Mn00607939_s1), the endogenous control. Comparative ΔΔCT method was used to determine fold change of human *OTOS* at each timepoint. Four independent experiments were performed and each sample was run in triplicate on a Life Technology Viia7 PCR machine.

RESULTS

Cohort Characteristics

Of 1,029 TCS who passed GWAS QC criteria, 608 (59.1%), 265 (25.8%), and 154 (15.0%) reported none, mild, and moderate/severe tinnitus, respectively. Subjects were dichotomized to cases (moderate/severe) and controls (none). Two cases were excluded for inconsistent responses. Cohort characteristics are provided in Table 1. Median age at diagnosis and evaluation was 31 (15–54) years and 39 (18–75) years, respectively. Median follow-up time was 5.2 (1–37) years. 91.6% were treated with either BEP (n=444, 58.3%) or EP (n=254, 33.3%). The remaining 64 (8.4%) received vinblastine (n=6), carboplatin (n=5) or ifosfamide (n=45) in combination with cisplatin, or had missing cisplatin dose data (n=12).

Diagnosis and Treatment Risk Factors

Cumulative cisplatin dose was significantly associated with tinnitus (OR per 100 mg/m^2=1.38, 95% confidence interval (CI): 1.1–1.7, P=0.007). Tinnitus risk for 400 mg/m^2-treated TCS (n=358) was not significantly greater than those receiving 300 mg/m^2 (n=300) (OR=1.14, 95% CI: 0.8–1.6, P=0.41). However, survivors who received >400 mg/m^2 (n=30, median dose=580 mg/m^2, range=402–828 mg/m^2) were at 2.61(95% CI: 1.8–3.9)-fold increased risk compared to those receiving ≤400 mg/m^2 (P<0.0001, Figure 1A). Tinnitus was not associated with cumulative doses of bleomycin (OR=1.01, P=0.13), etoposide (OR=1.02, P=0.33), or ifosfamide (OR=1.06, P=0.87) when cumulative cisplatin dose was included as a covariate. Three of five TCS receiving both carboplatin (median dose=1800 mg/m^2, range=5–3,646 mg/m^2) and cisplatin (median dose=200 mg/m^2, range=200–527

mg/m^2) had tinnitus (OR=7.37, 95% CI: 1.2–58.5, P=0.034). Tinnitus was associated with increasing age at diagnosis as a continuous variable (OR by decade=1.31, 95% CI: 1.1–1.6, P=0.006) and by quartile (OR=1.22, P=0.013, Figure 1B). Age at evaluation was also associated with tinnitus (OR=1.30, 95% CI: 1.1–1.5, P=0.002). No association with follow-up duration was observed (age-adjusted OR=0.99, P=0.57).

Associations with Self-Reported Health and Neuro-Ototoxic Symptoms

Self-reported overall health was significantly lower in cases than in controls (OR=0.54, 95% CI: 0.4–0.7, P<0.0001, Figure 2A). Tinnitus cases also reported using psychotropic medications more frequently than controls (OR=2.40, 95% CI: 1.3–4.4, P=0.003, Figure 2B). Audiometry was performed on 602 (78.4%) subjects. TCS with tinnitus had significantly worse hearing at every frequency examined (0.25 kHz–12 kHz, P<0.0001, Figure 3A). Tinnitus was also associated with self-reported hearing loss (OR=6.38, 95% CI: 4.9–8.5, P<0.0001, Figure 3B), problems hearing in a crowd (OR=8.28, 95% CI: 5.5–12.5, P<0.0001, Figure 3C), and persistent dizziness or vertigo (OR=6.40, 95% CI: 3.2–12.9, P<0.0001, Figure 3D), as well as symptoms of sensory neuropathy (OR range 1.66–2.72, P-values<0.0001, Figure 3E).

Associations with Modifiable Risk Factors

Tinnitus was significantly associated with hypertension (age-adjusted OR=2.07, 95% CI: 1.3–3.4, P=0.003), and hypercholesterolemia (age-adjusted OR=1.96, 95% CI: 1.2–3.2, P=0.009). In an age at clinical examination-adjusted model with both variables, tinnitus was significantly associated with hypertension (OR=1.77, 95% CI: 1.02–3.0, P=0.039), but not hypercholesterolemia (OR=1.6, 95% CI: 0.9–2.8, P=0.09). Tinnitus was associated with noise exposure at work (age-adjusted OR=1.93, 95% CI: 1.3–2.8, P=0.0005), and outside of work (age-adjusted OR=2.28, 95% CI: 1.6–3.3, P<0.0001). Risk was also associated with chronic (>15 years) smoking (age-adjusted OR=2.07, 95% CI: 1.2–3.8, P=0.005), but not increased among ever smokers (OR=1.26, 95% CI: 0.9–1.7, P=0.20) or with alcohol consumption (OR=0.92, 95% CI: 0.5–1.5, P=0.12). Association with chronic smoking remained significant after adjusting for age, hypertension, and noise exposure (OR = 1.79, 95% CI: 1.01–1.04, P=0.04).

Genome-Wide Association Study

Using GCTA's linear mixed model approach (26), we found that additive SNP effects explained a large fraction of CisIT variance (h^2=0.81±0.42, P=0.006). In GWAS, no SNP met genome-wide significance (Table S1, Figure S3). The first independent signal identified (rs7532231, OR = 0.45, P = 1.1×10^{-6}) is 116 kilobases upstream of the lncRNA *RP11–364B6.1*. The second (rs6671895, OR = 4.32, P = 1.2×10^{-6}) is intronic to the lncRNA *RP5-884C9.2*. The third (rs7606353, P=1.9×10^{-6}, Figure 4A) is a SNP 14 kilobases downstream of *OTOS* (otospiralin), a gene implicated in auditory function and survival of cochlear fibrocytes (33, 34). This variant is in near perfect linkage disequilibrium with rs74002277 in Europeans (CEU R^2=0.96), a SNP in the 3'-UTR region of *OTOS* and which alters several transcription binding motifs (35). The minor allele (G, 4% frequency) increased CisIT risk (OR=4.20, 95% CI: 2.3–7.5). Since there has been prior evidence that *OTOS* protects cochlear fibrocytes against cisplatin cytotoxicity (36), we hypothesized that

variants associated with higher *OTOS* expression lowered CisIT risk. In GTEx, *OTOS* was significantly expressed only in pituitary and thyroid tissues (Figure S4). We found that *OTOS* eQTLs were significantly enriched in GWAS independently from rs7606353 (P=0.018). One haplotype block containing six thyroid/pituitary eQTLs was driving this enrichment (Table S2, Figure S4). The minor allele (A) of the most significant variant within this block (rs10190781, 1.5% frequency) was associated with higher CisIT risk (OR=3.42, 95% CI: 1.5–9.5, P=0.007, Figure 4B) and lower *OTOS* expression (Figure 4C), suggesting that higher *OTOS* expression is protective. Tinnitus risk increased with the number of risk alleles in either locus (OR=3.77, 95% CI: 2.3–6.2, $P=9.5 \times 10^{-8}$, Figure 4D).

Functional Analysis

We tested the association between *OTOS* expression and cellular sensitivity to cisplatin (as measured by area under the dose response curve) in central nervous system (CNS) tumor lines (30, 31) and found a significant positive correlation (Spearman Rho=0.46, P=0.03; $R^2 = 0.25$, P = 0.01, Figure 4E), further supporting a protective *OTOS* function against cisplatin-induced damage. To examine the specificity of this correlation, we compared the sensitivity of CNS tumor cell lines to eight antineoplastic agents (5-fluorouracil (5-FU), bleomycin, bortezomib, cisplatin, cytarabine, docetaxel, etoposide, and vinblastine) based on *OTOS* expression levels (Table S3). We also examined the relationship between cisplatin sensitivity and *OTOS* expression in seven cancer cell line types (aerodigestive, breast, CNS, digestive system, lung, skin, and urogenital system; Table S4). Of the cytotoxic drugs tested, only cisplatin sensitivity demonstrated a statistically significant correlation with *OTOS* expression in CNS lines. Further, cisplatin sensitivity was associated with *OTOS* expression only in CNS tumor lines and not cell lines of other tissue origins. These data suggest a CNS tumor-specific association between *OTOS* expression and cisplatin sensitivity.

To functionally validate the role of *OTOS* gene expression in cellular sensitivity to cisplatin, we overexpressed *OTOS* in two cell lines, HEI-OC1 (a mouse auditory cell line) and L929 (a mouse fibroblast cell line), and compared gene expression levels and cellular proliferation following cisplatin treatment in *OTOS*-transfected cells and plasmid-transfected control cells (Figure 5A). Overexpression of human *OTOS* in HEI-OC1 resulted in increased cell viability following cisplatin treatment at 5, 7.5, 10, and 25 μM concentrations for 48 hours (P=0.004; Figure 5B) and 72 hours (P=0.008; Figure 5C). Figure S5 illustrates the effect of cisplatin on HEI-OC1 cells transfected with human *OTOS* and plasmid control. Furthermore, the cisplatin IC_{50} value of HEI-OC1 cells transfected with human *OTOS* (18.68 μM, 95% CI: 13.95–25.01 μM) was 1.8-fold higher than cells transfected with the control plasmid (10.66 μM, 95% CI: 6.04–18.82 μM) at 48 hours. Overexpression of *OTOS* was confirmed via qPCR, with human *OTOS* expression increasing 69-fold at the time of drug treatment, 24 hours following pOTOS transfection (Figure 5C inset). Similarly, overexpression of *OTOS* in L929 fibroblasts also markedly reduced cellular sensitivity to cisplatin following treatment for 48 hours (P=0.006; Figure 5D) and 72 hours (P=0.03; Figure 5E) concomitant with increased expression of OTOS (Figure 5E inset). The cisplatin IC_{50} value in *OTOS*-transfected cells (16.30 μM, 95% CI: 13.25–20.04 μM) was 1.5-fold higher than cells transfected with the control plasmid (10.96 μM, 95% CI: 8.82–13.61 μM) at 48 hours.

Pathway Analysis

We mapped SNPs to genes by proximity and performed pathway enrichment with Gene Ontology terms. We identified 134 coding genes with $P<5\times10^{-4}$ (Table S5). Ten pathways were significantly over-represented (Table S6), including *nervous system process* (q = 0.005), *potassium ion transmembrane transport* (q = 0.007), and *sensory perception of sound* (q = 0.005). Potassium transport most strongly implicated *KCNQ1* (rs2283200, $P = 8.9 \times 10^{-6}$), which maintains high potassium concentrations in the endolymph of the inner ear (37, 38).

DISCUSSION

To our knowledge, this is the first investigation to consider the effects of clinical, genetic, and lifestyle factors on CisIT risk. Here 25.8% and 15.2% of cisplatin-treated TCS reported mild and moderate/severe tinnitus at a median of 5 years after treatment. Survivors treated with 100–400 mg/m^2 cisplatin appeared to have similar CisIT risk, but those given >400 mg/m^2 were at 2.6-fold increased risk, suggesting a non-linear threshold-dose response. Follow-up time was not associated with CisIT, suggesting irreversibility, but longitudinal data are needed to support this observation. Significant associations with tinnitus were observed for hearing loss, vertigo, sensory neuropathy, poorer overall health, and use of psychotropic medications, as well as for hypertension, noise exposure, and chronic smoking. GWAS revealed independent signals implicating *OTOS* that had relatively large effect sizes (ORs of 3.5–4.5) and were relatively rare (minor allele frequencies 1–5%). We verified protective effects of *OTOS* through eQTL analysis and functional validation in two cell lines, demonstrating that decreased *OTOS* expression increases susceptibility to CisIT. Whether thyroid/pituitary eQTLs co-regulate expression in cochlear fibrocytes is unknown, and human cochlear transcriptomic databases are necessary for more precise interpretations. However, we provide further evidence for *OTOS's* protective functions *in silico* using central nervous system cell line data, and *in vitro* by overexpressing human *OTOS* in a mouse auditory cell line routinely used for *in vitro* drug ototoxicity screening (39) and a mouse fibroblast cell line. Pathway analysis implicated potassium transport genes, including the cochlear potassium transporter *KCNQ1*(37, 38).

Treatment-Related Risk Factors

Despite adequate statistical power, significant differences in tinnitus risk between TCS given 3 vs. 4 cycles of cisplatin-based chemotherapy were not evident. Brydøy et al. previously showed that TCS treated with >4 cycles of cisplatin-based chemotherapy had a 2.21-fold increased risk of tinnitus compared with those given 1–4 cycles (40), consistent with our findings. Bokemeyer et al. showed a dose-dependent increased risk for ototoxicity after cisplatin-based chemotherapy, but did not specifically assess tinnitus or compare by dosage group (41).

Modifiable Risk Factors

An approximate 2-fold association between hypertension and CisIT was apparent among our TCS. The relationship between hypertension and tinnitus in the general population is well-established, with a recent meta-analysis estimating a pooled OR of 1.37 (P<0.05) (42). It has

been hypothesized that hypertension causes damage to either the cochlear microcirculation and/or the stria vascularis, which supplies blood to the organ of Corti (43). This could explain the observed association.

Multiple studies have identified a relationship between tobacco use and tinnitus in the general population (44–46) and we found a significantly increased risk of tinnitus in survivors with >15 years of tobacco use. It is possible that long-term tobacco use may reduce peripheral blood flow, including to the inner ear microcirculation. The role of tobacco use in CisIT was examined by Brydøy et al., but significant associations were not apparent for either current or former smokers compared with never smokers (40), similar to our results.

Noise exposure is a known risk factor for tinnitus (47), but its role in the development of the hearing disorder in other cisplatin-treated cohorts has not been evaluated to our knowledge. Although Bokemeyer et al. reported an association between a history of noise exposure and cisplatin-induced ototoxicity, this term included both tinnitus and hearing loss (41).

Sensory Neuropathy

In our cohort, tinnitus was positively associated with symptoms of sensory neuropathy. This could suggest that inherent susceptibility to cisplatin-induced neurophysiological damage may partially underlie various neuro-otological symptoms, possibly involving pharmacokinetic pathways, intrinsic cisplatin sensitivity, reduced regenerative capacity, or altered neural plasticity. Another possible explanation is that of patient perception. Self-reported tinnitus, hearing loss, and neuropathy in cancer survivors have been linked to the Impact of Events Scale-Revised (IES-R) hyperarousal scores (13), suggesting that differences in perception and appraisal could partially explain our findings. One study showed that audiometric thresholds correlated with tinnitus awareness time and loudness, but that tinnitus-induced annoyance was associated with proneness to exhibit anxious responses (48, 49). These investigations highlight how both cochlear and central nervous mechanisms contribute to tinnitus.

Tinnitus Pathophysiology

While single-variant analysis did not identify significant genetic signals, we found that cumulatively, additive SNP effects explain a large portion of variance in CisIT, further supporting the hypothesis that CisIT is a polygenic trait with complex genetic underpinnings (4). Several mechanisms of tinnitus have been proposed, but no consensus on its etiology has been reached. One model postulates that disruptions to the endocochlear potential result in inhibitory-excitatory imbalances affecting spontaneous cochlear activity and lead to tinnitus (50). Inner-ear fibrocytes supply potassium ions to strial cells which pump them into the endolymph to maintain the endocochlear potential, the “cochlear battery” driving auditory transduction (51). The link between endocochlear potential and spontaneous auditory nerve activity has been established (52). Our GWAS points to the potential importance of this hearing mechanism in CisIT.

OTOS mRNA and protein expression localize to spiral limbus and spiral ligament fibrocytes (33), and overexpression protects primary spiral ligament fibrocytes against cisplatin

cytotoxicity (36). In support of the protective effect of *OTOS* against cisplatin sensitivity, our *in silico* analysis indicated that cisplatin sensitivity is positively associated with increased *OTOS* expression in CNS tumor cell lines, a rough approximation of cell types that would be affected by cisplatin-induced neurotoxicity. While CNS tumor cell lines are not an ideal proxy for assessing toxicity to inner ear cells, we further validated the protective effects of *OTOS* by demonstrating that its overexpression *in vitro* reduces cisplatin sensitivity in two cell lines (HEI-OC1 and L929), with HEI-OC1 more accurately reflecting inner ear cells that are adversely affected by cisplatin.

In support of our findings, two prior studies have elucidated the importance of *OTOS* expression in mammalian hearing. Inducible knockdown of *OTOS* in guinea pigs caused severe degeneration of the organ of Corti and deafness, along with damage to fibrocytes (33). Interestingly, although *Otos* $^{-/-}$ mice had damaged fibrocytes, they did not exhibit histological abnormalities of sensory hair cells, and experienced only moderate deafness (34). Delprat et al. attributed this difference to long-term adaptation to congenital *Otos* absence (33). Therefore, it is plausible that inducible knockdown, modeling key aspects of direct auditory insults such as cisplatin administration, will acutely alter the ionic composition of the endolymph, disrupting hair cell metabolism and survival.

Breglio et al. recently showed a reduction of endocochlear potential following cisplatin administration in mice, and preferential accumulation of cisplatin in the stria vascularis (53). We linked potassium transport to CisIT in our pathway analysis, which most strongly implicated *KCNQ1*, an important cochlear ion channel. This voltage-gated channel localizes to marginal cells of the stria vascularis where it maintains high K+ concentrations in the endolymph (37). Mutations in *KCNQ1* result in deafness and long QT syndrome (54).

Taken together, these findings point to the function of supporting cells in the cochlear lateral wall in cisplatin ototoxicity and hair cell survival. Impaired maintenance of the endolymph causes hair cell death and permanent hearing loss (55), which may be partially mediated by a reduced endocochlear potential. However, prior research has largely focused directly on cisplatin's cytotoxic mechanisms in hair cells (56). Our new findings, together with those in the literature, suggest that novel strategies aimed at protecting supporting cells in the cochlear lateral wall, perhaps by stimulating *OTOS* expression, may prove beneficial. Very little is known about the protein product of the gene, its functions, and its interactions. Better understanding of the functional protein and its cellular localization and interactions is warranted. A potential approach would be to use a gene expression-based high throughput screen to identify small molecules or siRNA capable of markedly increasing *OTOS* expression without perturbing the viability of auditory cells, such as HEI-OC1. The identification of compounds or siRNAs resulting in increased OTOS expression could be evaluated for their effect on protecting cells from cisplatin damage. Importantly, the utility of this approach depends on the modulation not impacting the antineoplastic activity of cisplatin.

It remains unclear whether the effects of *OTOS* on tinnitus occur directly or are mediated by effects on hearing loss. However, if *OTOS* protects against tinnitus indirectly by preventing hearing loss, it remains a viable candidate for cisplatin otoprotection. It is also unclear

whether the actions of *OTOS* are uniquely pertinent to cisplatin or generalizable to other ototoxic insults like noise, although evidence of direct protection against cisplatin cytotoxicity is presented here and in the literature (36). Future studies should elucidate the cellular functions and interactions of otospiralin to assess its viability as a target for cisplatin otoprotection.

CONCLUSIONS

Strengths of our study include the collection of detailed treatment data, homogeneous cisplatin-based chemotherapy, long-term follow-up, quantitative hearing evaluations, the use of validated instruments to assess neurotoxic symptoms, and the novel implementation of genome-wide scans to nominate cellular mechanisms and targets. Our results point to cochlear mechanisms that have not yet been widely explored in cisplatin ototoxicity, and may provide novel opportunities for targeted otoprotection.

An intrinsic limitation of all cross-sectional studies is the inability to make causal inferences. Further, questionnaires with more detailed information on the experience of tinnitus, including baseline measurements pre-chemotherapy, would provide more conclusive evidence with regard to associations noted here. However, given the young median age of patients at treatment, it is unlikely that tinnitus was pre-existing. It is important to note that the results of our translational research could potentially impact millions of patients worldwide annually diagnosed with cisplatin-treated cancers who are at risk for CisIT. Our findings may also inform tinnitus research in non-platinum-treated patients. In view of our results, health care providers can improve management of cancer survivors experiencing CisIT by recommending monitoring of blood pressure, encouragement of smoking cessation, and avoidance of additional ototoxic insults such as noise exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

CCLE	Cancer Cell Line Encyclopedia
CIPN20	Chemotherapy Induced Peripheral Neuropathy 20 item questionnaire
CisIT	cisplatin-inducted tinnitus

eQTL	expression quantitative trait loci
GCT	germ cell tumor
GWAS	genome-wide association study
SCIN	Scale for Chemotherapy-Induced Neurotoxicity
SNP	single nucleotide polymorphism
TCS	testicular cancer survivors
QoL	quality of life

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TRANSLATIONAL RELEVANCE

Cisplatin is one of the most commonly prescribed chemotherapeutics worldwide, but elicits several debilitating off-target toxicities, including cisplatin-induced tinnitus (CisIT; a perceived ringing/buzzing noise with no external sound present). CisIT significantly affects perceived quality of life, but its etiology remains poorly understood. We found that cumulative cisplatin doses exceeding 400 mg/m^2 to markedly increase CisIT susceptibility, and that survivors with tinnitus were more likely to experience hearing loss, vertigo, and problems hearing in a crowd, as well as report poorer health and greater reliance on psychotropic medication. Through a genome-wide association study, we identified genetic variants in *OTOS*, a gene vital for the maintenance of normal hearing, to be associated with CisIT, and further demonstrated that increased *OTOS* expression was associated with reduced cisplatin sensitivity *in silico* and *in vitro*. These data provide novel mechanistic insights into CisIT susceptibility, and may inform clinicians of potential risk factors and comorbidities.

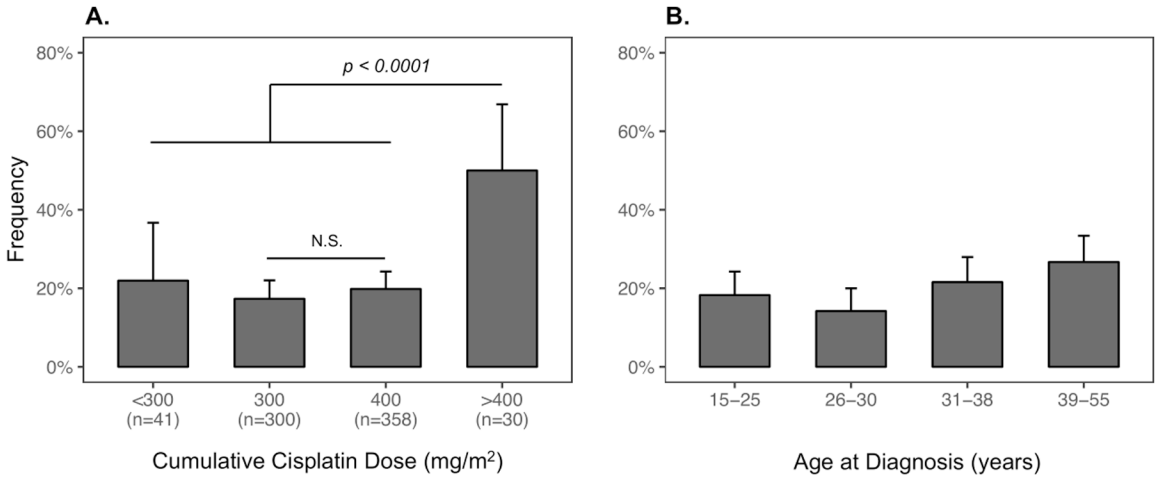

Figure 1. Tinnitus Frequency by Cumulative Cisplatin Dose and Age at Testicular Cancer Diagnosis (quartiles).

A. Bar plot showing the frequency of tinnitus by cumulative cisplatin dose group (<300, 300, 400, and >400 mg/m^2) illustrates that the significant association between dose and tinnitus (logistic regression OR = 1.38, 95% CI: 1.1–1.7, P=0.007) is due to the significantly increased risk of tinnitus in patients treated with doses > 400 mg/m^2 (post-hoc Chi-squared P < 0.0001). The number of subjects per category is presented on the x-axis under the dose group label. **B.** Bar plot representing the frequency of tinnitus by age at diagnosis quartile shows positive association with cisplatin risk (OR by decade = 1.31, 95% CI: 1.1–1.6, P = 0.006). Error bars represent the binomial 95% CIs for each subgroup. N.S = not significant.

Figure 2. Self-reported Health and Use of Psychotropic Medications by Tinnitus Status.

A. Bar plot of the distribution of self-reported health according to tinnitus status. Patients were asked: "How would you rate your overall health?" and responded on a poor-excellent ordinal scale, which was converted to a numeric scale (0 = poor/fair, 1 = good, 2 = very good, 3 = excellent). Logistic regression revealed significant negative correlation between tinnitus status and self-reported health (OR=0.54, 95% CI: 0.4–0.7, P<0.0001). **B.** Bar plot of psychotropic medication use by tinnitus status shows significantly higher prevalence of psychotropic medication use in tinnitus cases (OR= 2.4, 95% CI: 1.3–4.4, P=0.003). Patients were dichotomized to "Yes" and "No" psychotropic medication use based on receiving medications from a list of frequently prescribed antidepressants, anxiolytics, and antipsychotics (Supplemental Methods, (15)). The number of subjects per category is presented on the x-axis. Error bars represent the binomial 95% CIs for each subgroup.

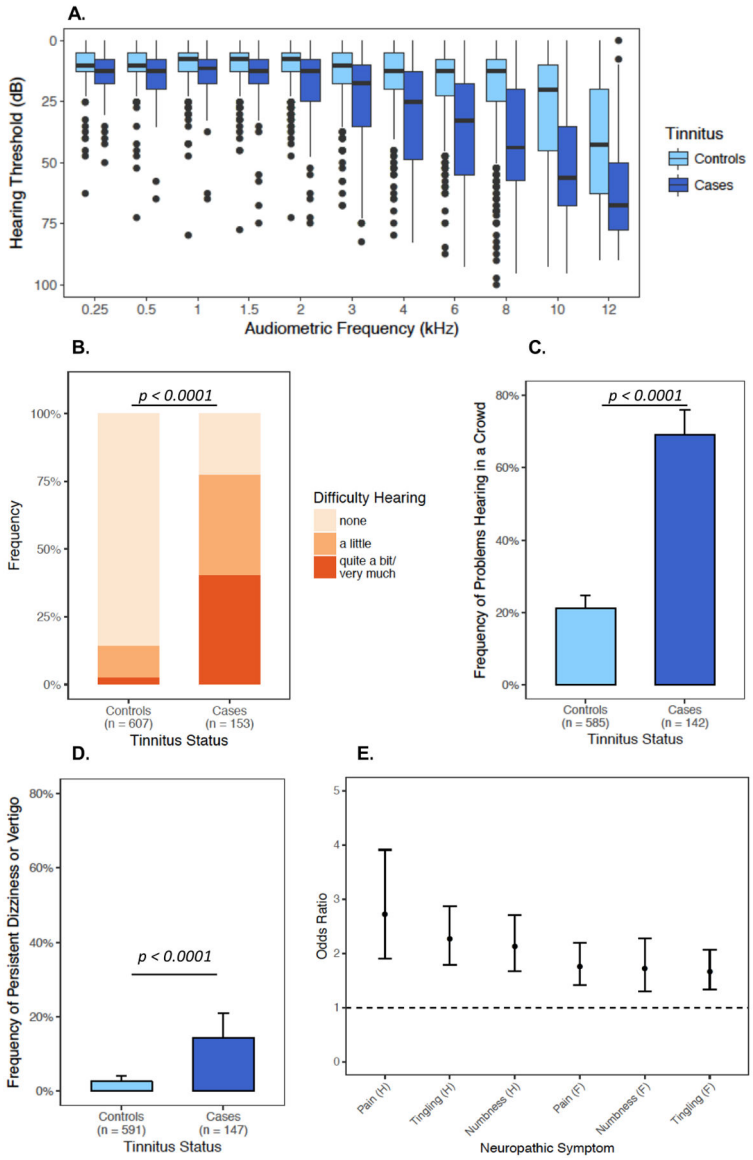

Figure 3. Associations of Tinnitus with Subjective and Objective Hearing Loss, Problems Hearing, Vertigo, and Neurological Symptoms.

A. Box plot of audiometric hearing thresholds (y-axis, dB) across all tested audiometric frequencies (x-axis, kHz) showing significantly worse hearing in tinnitus cases at each frequency ($P < 0.0001$ at each frequency). Box centers indicate medians, hinges indicate interquartile regions (IQRs), and whiskers indicate 1.5x IQRs. Points outside the range of 1.5x IQR are shown. Bar plots of self-reported **B.** difficulty hearing (OR = 6.36, 95% CI: 4.8–8.5, $P < 0.0001$), **C.** problems hearing in a crowd (OR = 8.2, 95% CI: 5.5–12.5, $P<0.0001$), and **D.** persistent dizziness or vertigo (OR = 6.40, 95% CI: 3.2–12.9, $P<0.0001$). Error bars represent the binomial 95% CIs for subgroup. **E.** Forest plot showing the odds ratio (OR, center points) and 95% confidence intervals (error bars) of the association between tinnitus and neurotoxic symptoms from the EORTC-CIPN20. “H” denotes hands/ fingers. “F” denotes feet/toes. Responses to EORTC-CIPN20 items were converted from a

none-very much Likert scale to a numerical ordinal scale (0–3). Associations in B-E were evaluated using logistic regression adjusted for age at diagnosis.

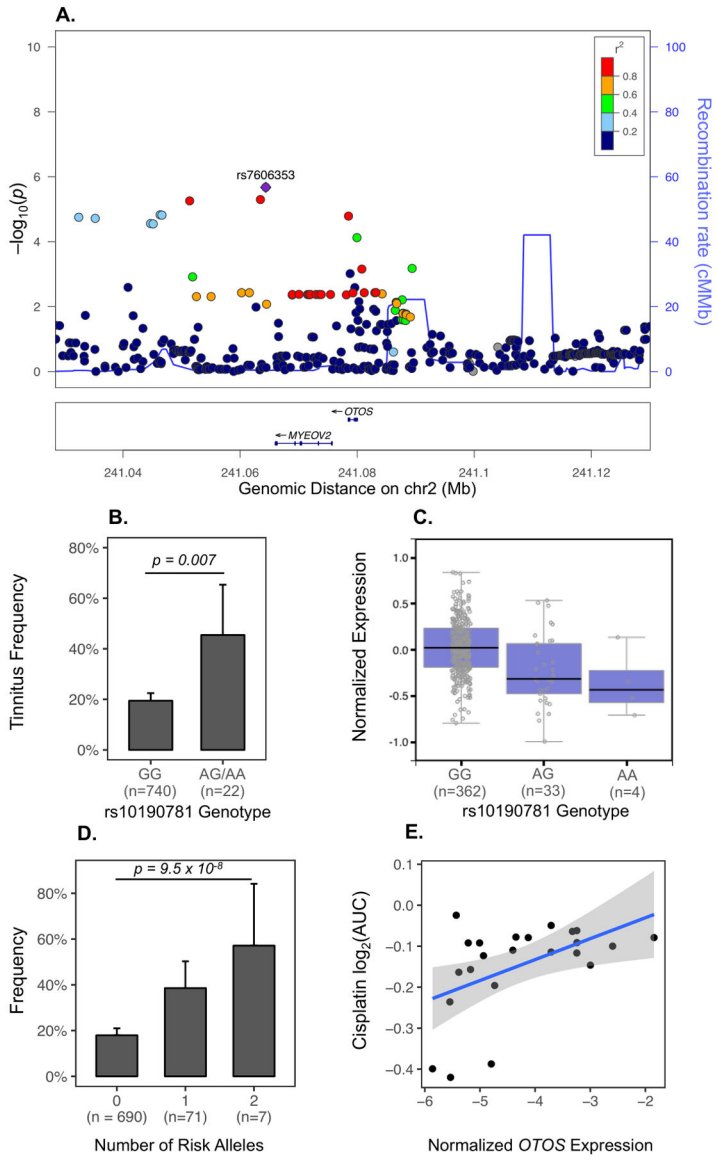

Figure 4). GWAS of Cisplatin-Induced Tinnitus Reveals Genetic Loci Near *OTOS* gene.

A. LocusZoom plot of the third most significant GWAS signal near two adjacent genes: *OTOS* and *MYEOV2*. Each point represents a SNP. The x-axis indicates chromosomal position. The left y-axis shows $-\log_{10}$(p-value) of association with CisIT and the right y-axis and blue line indicate the recombination rate in centimorgans/megabase (cM/MB). The color of each variant indicates the linkage disequilibrium R^2 with the top signal in the region, rs7606353 (purple). **B.** Bar plot of CisIT frequency by genotype of *OTOS* eQTL, rs10190781. The minor allele (G) increases CisIT risk (OR = 3.42, P = 0.007). Error bars represent the binomial 95% CIs. **C.** Boxplot of *OTOS* expression in thyroid by rs10190781 genotype indicates that the minor allele is associated with lower expression of *OTOS*. Data were obtained from the GTEx Portal on 04/28/18 **D.** Bar plot of number of risk alleles for both rs7606353 and rs10190781 shows additive allele effects (OR = 3.77, 95% CI: 2.3–6.2], $P=9.5 \times 10^{-8}$. **E.** Scatter plot of cisplatin resistance as a function of normalized *OTOS*

expression. Cisplatin resistance, measured as the area under the cisplatin dose-response curve, for all central nervous system tumor lines (19 glioma and 4 neuroblastoma lines) was extracted from CancerRX and normalized *OTOS* expressions were downloaded from the Cancer Cell Line Encyclopedia. Correlation was assessed non-parametrically using Spearman's Rank method (Rho = 0.46, P = 0.03).

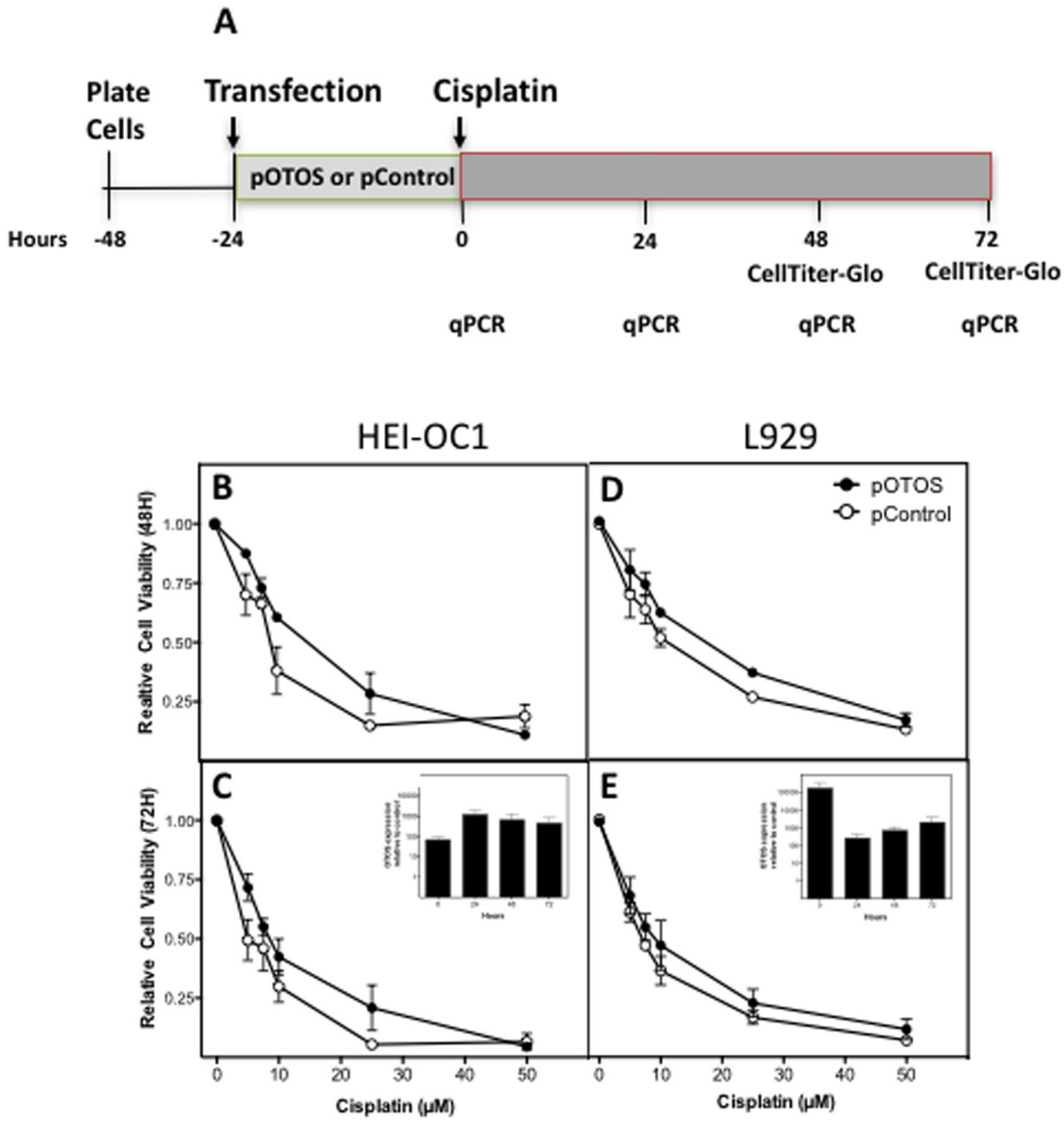

Figure 5). Effects of *OTOS* Overexpression on Cisplatin Sensitivity in HEI-OC1 Mouse Cochlear and L929 mouse fibroblast cell lines.

A. Experimental scheme of transfection and cisplatin treatment, followed by cell viability and qPCR analysis. Cells were transfected with human *OTOS* (pOTOS) or a control plasmid (pControl) for 24 hours prior to cisplatin treatment. **B.** Cell viability of HEI-OC1 cells transfected with either pOTOS (closed circles) or the control plasmid (pControl; open circles) following treatment with increasing concentrations of cisplatin for 48 hours (P=0.004) or **C.** 72 hours (P=0.008)**. Inset:** Human *OTOS* mRNA expression in HEI-OC1 cells corresponding to 0, 24, 48, and 72 hours after cisplatin treatment (24, 48, 72, and 96 hours after transfection) depicted as pOTOS relative to pControl. **D.** Cell viability of L929 fibroblasts transfected with either pOTOS (closed circles) or the control plasmid (pControl; open circles) following treatment with increasing concentrations of cisplatin for 48 hours (P=0.006) or **E.** 72 hours (P=0.03)**. Inset:** Human *OTOS* mRNA expression in L929 fibroblasts corresponding to 0, 24, 48, and 72 hours after cisplatin treatment (24, 48, 72, and

96 hours after transfection) depicted as pOTOS relative to pControl. Data shown are from 3 independent experiments, with each concentration or time point within an experiment evaluated in duplicate or triplicate. Error bars reflect S.E.M. Statistical significance was determined using 2-way ANOVA analysis with Sidak correction for multiple comparisons.

Table 1.

Demographic Features, Clinical Characteristics, and Patient-Reported Outcomes of 762 Male Germ Tumor Survivors According to Tinnitus Status

Characteristic	All Participants (n=762)	Tinnitus Controls (n=608)	Tinnitus Cases[a] (n=154)
Age at diagnosis (years)[b]			
Median (range)	31 (15–54)	30 (15–54)	35 (16–52)
<20	43 (5.7%)	35 (5.8%)	8 (5.2%)
20–29	300 (39.8%)	249 (41.5%)	51 (33.1%)
30–39	243 (32.2%)	196 (32.7%)	47 (30.5%)
40–55	168 (22.2%)	120 (20.0%)	48 (31.2%)
Age at assessment (years)			
Median (range)	39 (18–75)	38 (18–71)	43 (20–75)
<20	6 (0.8%)	5 (0.8%)	1 (0.6%)
20–29	134 (17.6%)	115 (18.9%)	19 (12.3%)
30 to 49	274 (36.0%)	230 (37.8%)	44 (28.6%)
40 to 49	202 (26.5%)	150 (24.7%)	52 (33.8%)
50 to 59	123 (16.1%)	88 (14.5%)	35 (22.7%)
≥60	23 (3.0%)	20 (3.3%)	3 (2.0%)
Time since therapy completion (years)[c]			
Median (range)	5.2 (1–37)	5.1 (1–37)	6.2 (1–35)
≤1	3 (0.4%)	1 (0.2%)	2 (1.3%)
>1 and ≤5	362 (48.0%)	293 (48.8%)	69 (44.8%)
>5 and ≤10	180 (23.9%)	145 (24.2%)	35 (22.7%)
>10 and ≤20	159 (21.1%)	127 (21.2%)	32 (20.8%)
>20	50 (6.6%)	34 (5.7%)	16 (10.4%)
Educational level			
Secondary school or less	95 (12.6%)	62 (10.3%)	33 (21.9%)
Post-secondary school training	166 (22.0%)	129 (21.4%)	37 (24.5%)
College graduate	334 (44.2%)	276 (45.7%)	58 (38.4%)
Post graduate training/degree	160 (21.2%)	137 (22.7%)	23 (15.2%)
Chemotherapy regimen[d]			
BEP	444 (58.3%)	355 (58.4%)	89 (57.8%)
EP	254 (33.3%)	205 (33.7)	49 (31.8%)
Other	64 (8.4%)	48 (7.9%)	16 (10.4%)
Number of cycles, platinum-based chemotherapy[e]			
≤2	15 (2.0%)	13 (2.2%)	2 (1.3%)
3	333 (44.0%)	272 (45.1%)	61 (39.6%)
4	389 (51.4%)	307 (50.9%)	82 (53.2%)

Characteristic	All Participants (n=762)	Tinnitus Controls (n=608)	Tinnitus Cases[a] (n=154)
>4	20 (2.6%)	11 (1.8%)	9 (5.8%)
Cumulative cisplatin dose (mg/m^2)[f]			
Median	400 (100–828)	400 (200–800)	400 (100–827.6)
< 300	41 (5.5%)	32 (5.4%)	9 (5.9%)
300	300 (40.0%)	248 (41.5%)	52 (34.0%)
>300 and <400	21 (2.8%)	15 (2.5%)	6 (3.9%)
400	358 (47.7%)	287 (48.1%)	71 (46.4%)
> 400	30 (4.0%)	15 (2.5%)	15 (9.8%)
Reduced hearing in past four weeks[g]			
Not at all	555 (73.1%)	521 (85.7%)	34 (22.5%)
A little	127 (16.7%)	71 (11.7%)	56 (37.1%)
Quite a bit/very much	77 (10.1%)	16 (2.6%)	61 (40.4%)
Problems hearing in crowds[h]			
Yes	222 (30.6%)	124 (21.2%)	98 (69.0%)
No	505 (69.4%)	461 (78.8%)	44 (31.0%)
Requires hearing aid[i]			
Yes	2 (0.3%)	2 (0.3%)	0 (0%)
No	741 (99.7%)	602 (99.7%)	139 (100%)
Noise exposure			
None	430 (56.4%)	365 (60.0%)	65 (42.2%)
Work related OR other exposure	213 (28.0%)	163 (26.8%)	50 (32.5%)
Work related AND other exposure	119 (15.6%)	80 (13.2%)	39 (25.3%)
Audiometrically assessed hearing loss[j,k]			
None/mild	238 (39.4%)	220 (45.4%)	18 (15.0%)
Moderate	93 (15.4%)	75 (15.5%)	18 (15.0%)
Moderately severe	129 (21.4%)	103 (21.3%)	26 (21.7%)
Severe/profound	144 (23.8%)	86 (17.8%)	58 (48.3%)
Peripheral neuropathy[l]			
None	373 (49.0%)	329 (54.1%)	44 (28.6%)
Mild	281 (36.9%)	215 (35.4%)	66 (42.9%)
Severe	108 (14.1%)	64 (10.5%)	34 (28.6%)
Persistent vertigo or dizziness[m]			
Yes	36 (4.9%)	15 (2.5%)	21 (14.3%)
No	702 (95.1%)	576 (97.5%)	126 (85.7%)
Self-reported health[n]			
Excellent	134 (17.6%)	121 (20.0%)	13 (11.7%)
Very good	317 (41.7%)	264 (43.6%)	53 (45.5%)

Characteristic	All Participants (n=762)	Tinnitus Controls (n=608)	Tinnitus Cases[a] (n=154)
Good	266 (35.0%)	196 (32.3%)	70 (34.4%)
Poor/fair	43 (5.7%)	25 (4.1%)	18 (8.4%)
Medication for anxiety, psychosis, and/or depression[o]			
Yes	57 (10.1%)	38 (8.3%)	19 (17.75%)
No	510 (89.9%)	422 (91.7%)	88 (82.2%)
Hypertension and on medication			
Yes	106 (14.0%)	69 (11.6%)	37 (24.2%)
No	651 (86.0%)	535 (89.9%)	116 (75.8%)
Smoking status[p]			
Ever smoker	303 (39.9%)	235 (38.8%)	68 (44.2%)
Never smoker	457 (60.1%)	371 (61.2%)	86 (55.8%)
Alcohol consumption[q]			
≤4 drinks per week	487 (64.2%)	384 (63.4%)	103 (66.9%)
5–7 drinks per week	172 (22.7%)	140 (24.3%)	32 (20.8%)
≥2 drinks per day	100 (13.2%)	81 (12.3%)	19 (12.3%)

Abbreviations: BEP, bleomycin, etoposide, and cisplatin, EP, etoposide, and cisplatin, NA not applicable

NOTE: Data presented as number (%) unless otherwise noted

[a]Restricted to patients who reported “quite a bit’ or ‘very much” tinnitus. Patients who reported “a little” tinnitus (n=265) are excluded from the table and all analyses.

[b]Category excludes 8 participants for whom age at diagnosis was not recorded.

[c]Category excludes 8 participants for whom this variable was not stated.

[d]*BEP* category includes patients who received only bleomycin, etoposide, and cisplatin; *EP* includes patients who received only etoposide and cisplatin. The *other* category includes patients who received other antineoplastic agents, including ifosfamide (n=45), vinblastine (n=6), and carboplatin (n=5), or who were missing dose data (n=12).

[e]Category excludes 5 participants for whom number of cycles was not stated.

[f]Category excludes 12 participants for whom complete dose data were not available.

[g]Category excludes 3 participants who did not answer this question.

[h]Category excludes 35 participants who did not answer this question.

[i]Category excludes 19 participants who did not answer this question.

[j]Category excludes 158 participants who did not complete audiometric testing.

[k]ASHA criteria defined hearing loss severity as the following: mild: 21 to 40 dB; moderate: 41 to 55 dB; moderately severe: 56 to 70 dB; severe: 71 to 90 dB; and profound: > 90 dB; for at least one tested frequency for either ear (https://www.asha.org/public/hearing/Degree-of-Hearing-Loss)

[l]Following conversion of the Likert scale: “none, a little, quite a bit, very much” to a 0–3 numeric scale, each individual was assigned a summary statistic for the sensory subscale (Cronbach ($\alpha = 0.88$) and the motor subscale ($\alpha = 0.78$) by taking the mean of the response in the subscale: none (mean = 0), mild ($0 < \text{mean} \leq 1$), severe (mean > 1) (16, 17).

[m]Category excludes 24 participants who did not answer this question.

[n] Category excludes 2 participants who did not answer this question.

[o] Category excludes 195 who did not answer this question. Participants could report more than one psychotropic medication including anxiolytics, antipsychotics, and antidepressants. Psychotropic medications investigated include: alprazolam (n=8), aripiprazole (n=2), amphetamine + dextroamphetamine (n = 14), amantadine (n = 1), buproprion (n = 9), carbidopa/levodopa (n = 1), citalopram (n = 8), clonazepam (n = 13), desvenlafaxine (n = 1), diazepam (n = 1), duloxetine (n = 8), escitalopram (n = 19), fluoxetine (n = 5), fluvoxamine (n = 1), hydroxazine (n = 1), lisdexamfetamine (n = 4), lorazepam (n = 1), methylphenidate (n = 13), nortriptyline (n = 2), olanzapine (n = 2), paroxetine (n = 5), pramipexole (n = 1), sertraline (n = 7), trazodone (n = 8), valproate (n = 1), venlafaxine (n = 10).

[p] Category excludes 2 participants for whom this outcome was not stated.

[q] Category excludes 3 participants for whom this outcome was not stated.