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# Common variants in *ACYP2* influence susceptibility to cisplatin-induced hearing loss

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**Taking a genome-wide association study approach, we identified inherited genetic variations in *ACYP2* associated with cisplatin-related ototoxicity (rs1872328: $P = 3.9 \times 10^{-8}$, hazard ratio = 4.5) in 238 children with newly diagnosed brain tumors, with independent replication in 68 similarly treated children. The *ACYP2* risk variant strongly predisposed these patients to precipitous hearing loss and was related to ototoxicity severity. These results point to new biology underlying the ototoxic effects of platinum agents.**

Cisplatin is one of the most widely used anticancer agents, with well-documented efficacy against a variety of solid tumors in children and adults[1–3]. However, platinating agents are also known for debilitating adverse effects. Particularly in children, cisplatin-related hearing loss negatively affects quality of life and severely impedes language development, with irreversible long-term effects[4]. With cumulative cisplatin dosages at or above 400 mg/m$^2$, the auditory impairment is typically bilateral and highly prevalent, with up to 70% of children suffering severe hearing loss necessitating hearing aids[5]. The proposed mechanism of ototoxicity involves the generation and release of both proapoptotic factors and free radicals within the sensory outer hair cells of the cochlea upon exposure to cisplatin[4]. Although cisplatin is the most ototoxic, this adverse effect is not completely avoided by the use of other platinum agents (for example, carboplatin[6,7]), and substitution is rarely performed when cisplatin is indicated for treatment of a patient's cancer owing to concerns of inferior efficacy and/or prolonged myelosuppression from equivalent doses of carboplatin[8].

Younger age and concurrent craniospinal irradiation have been reported to increase the risk of cisplatin-related ototoxicity[5,9,10]. However, inter-patient variability is remarkable even within highly uniform treatment regimens[8,11,12], and an inherited genetic predisposition is hypothesized[5,13]. Many potential candidate genes have been investigated with largely inconsistent results, plausibly because of non-uniform patient populations, heterogeneous and non-protocol-based platinum therapies and/or inadequate and inconsistent audiometric monitoring[14]. Although no genetic risk variants have been definitively linked to cisplatin-related hearing loss, the potential impact of cisplatin pharmacogenomics should not be underappreciated. Identification of the genetic basis of cisplatin-related ototoxicity could lead to an improved mechanistic understanding, advance protective interventions and facilitate the development of less ototoxic therapies.

To this end, we sought to perform a genome-wide association study (GWAS) to comprehensively discover germline SNPs associated with cisplatin-related ototoxicity, in the context of frontline clinical treatment protocols of children with embryonal brain tumors.

The discovery GWAS included 238 children treated for newly diagnosed embryonal brain tumors on the St. Jude medulloblastoma 96 and 03 protocols (referred to as SJMB96 and SJMB03, respectively, hereafter; **Supplementary Figs. 1** and **2**), for whom hearing loss was prospectively monitored with a predefined schedule[15]. Ototoxicity primarily occurred between 1 and 6 months after the start of cisplatin therapy (**Supplementary Fig. 3**). Sixty-one percent of the children developed detectable ototoxicity (Chang score > 0), and 37% experienced clinically relevant ototoxicity (Chang score ≥ 2a; **Supplementary Table 1**). Younger age at diagnosis and higher dose of craniospinal irradiation were significantly associated with increased risk of hearing loss (**Table 1**). The frequency of ototoxicity decreased in the SJMB03 protocol in comparison to the earlier SJMB96 treatment regimen, plausibly because of the reduced target volume of

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**Table 1 Association of patient characteristics with cisplatin ototoxicity in the discovery GWAS cohort**

| Feature | Ototoxicity status: Chang score = 0 ($n$ = 93) | Ototoxicity status: Chang score > 0 ($n$ = 145) | $P$ value (time to event) |
|---|---|---|---|
| | Mean ± s.d. | | |
| Age at diagnosis (years) | 10.0 ± 4.3 | 8.5 ± 3.8 | **0.014** |
| Cisplatin cumulative dosage (mg/m$^2$) | 288.7 ± 36.1 | 286.5 ± 34.8 | 0.30 |
| | Number of patients (%) | | |
| Sex | | | |
| Female | 36 (40.0) | 54 (60.0) | 0.71 |
| Male | 57 (38.5) | 91 (61.5) | |
| Craniospinal irradiation | | | |
| <25 Gy | 78 (48.4) | 83 (51.6) | **<0.0001** |
| ≥25 Gy | 15 (19.5) | 62 (80.5) | |
| Treatment protocol | | | |
| SJMB03 | 87 (42.0) | 120 (58.0) | **0.013** |
| SJMB96 | 6 (19.4) | 25 (80.6) | |
| Tumor location | | | |
| Infratentorial | 78 (37.3) | 131 (62.7) | 0.22 |
| Supratentorial | 15 (51.7) | 14 (48.3) | |
| | Median (min, max) | | |
| Genetic ancestry | | | |
| PC1 | −11.8 (−19.0, 63.3) | −12.3 (−18.5, 62.0) | 0.82 |
| PC2 | −1.7 (15.7, 42.0) | −2.1 (−19.4, 40.5) | 0.20 |
| PC3 | −0.1 (−15.2, 17.8) | −0.6 (−20.5, 20.8) | 0.39 |

$P$ values were estimated using the Cox regression model, with ototoxicity treated as a time-dependent variable. $P$ values <0.05 are shown in bold. Genetic ancestry was determined by genome-wide SNP genotype using EIGENSTRAT (PC, principal component).

craniospinal irradiation and/or the use of amifostine. Sex, genetic ancestry, cumulative cisplatin dosage and tumor location did not significantly influence ototoxicity (**Table 1**).

As quality control before GWAS, we first removed variants that were poorly genotyped (call rate < 98%) or rare (minor allele frequency (MAF) < 1%). The final GWAS data set included genotypes at 1,716,999 variants in 238 children treated with cisplatin chemotherapy (Online Methods and **Supplementary Figs. 2** and **4**). Treating hearing loss as a time-dependent variable, we compared the frequency and onset of hearing loss (Chang score > 0) among patients with different genotypes at each SNP. After adjusting for genetic ancestry, age at diagnosis, craniospinal irradiation dose (<25 Gy or ≥25 Gy) and study protocol (SJMB96 or SJMB03), rs1872328 within the *ACYP2* gene on chromosome 2p16.2 showed the strongest association signal ($P = 3.9 \times 10^{-8}$, hazard ratio (HR) = 4.50 with 95% confidence interval (CI) = 2.63–7.69; **Fig. 1a** and **Supplementary Data**). A subsequent permutation test confirmed that the association at rs1872328 was stronger than what would be expected by chance (permutation $P = 2 \times 10^{-6}$). We observed no other genome-wide significant loci. A second SNP (rs7604464: $P = 1 \times 10^{-7}$, HR = 3.81 (2.33–6.24)) in *ACYP2* approached genome-wide significance (**Supplementary Table 2**), with a total of 16 SNPs within a 300-kb window at this locus showing nominal associations ($P < 0.05$; **Supplementary Fig. 5**). With conditioning on rs1872328, three SNPs within this region maintained significant associations (rs1569087, rs13396318 and rs6724542; $P < 0.05$), suggesting independent contributions to ototoxicity. Thirty-three SNPs showed at least suggestive evidence for association with ototoxicity in the discovery GWAS ($P < 5 \times 10^{-6}$; **Supplementary Table 2**).

All (100%) of the 20 patients carrying the A allele at rs1872328 developed ototoxicity, regardless of whether the patient was heterozygous or homozygous for the risk-associated allele. In contrast, ototoxicity was noted in 57.3% of children who did not carry this risk allele. More notably, hearing loss in children with the AA or AG genotype occurred in a particularly precipitous fashion in comparison to children with the GG genotype (**Fig. 1b**). Although the risk variant at rs1872328 was more common in individuals of African descent, the association remained highly significant when we restricted the analyses to European Americans ($P = 0.001$, HR = 3.85 (1.72–8.33); **Supplementary Fig. 6**). A strong correlation was also noted between genotype at rs1872328 and the severity of ototoxicity ($P = 0.0005$), with the risk allele frequency increasing gradually with the clinical grade of ototoxicity. The association at the *ACYP2* SNP was consistent regardless of the irradiation dose and across clinical protocols (**Supplementary Fig. 6**).

To validate the association at the *ACYP2* locus, we genotyped rs1872328 and rs7604464 in young children (<3 years old) with brain tumors treated on the St. Jude YC07 protocol (SJYC07; **Supplementary Fig. 7** and **Supplementary Table 3**). In comparison to the discovery GWAS cohort, patients on the SJYC07 protocol received the same cisplatin regimen but reduced and delayed irradiation in consideration of their young age. Of the 68 SJYC07 patients with evaluable genotype and hearing assessment, the association was validated via the same time-to-event approach used in our discovery cohort ($P = 0.006$, HR = 2.94 (1.35–6.25)). Four patients carried the risk allele with a heterozygous genotype at rs1872328 (no AA genotype was observed), all of whom developed ototoxicity (**Fig. 1c**). Similar to the results in the discovery GWAS, children with the A allele suffered rapid hearing impairment in comparison to those who did not carry the risk allele.

To further identify ototoxicity-related variants at the *ACYP2* locus, we resequenced the exonic region of *ACYP2* in 257 children included in the discovery and validation cohorts (**Supplementary Fig. 8**). We observed a total of four variants in nine patients: one missense (singleton), one silent (recurrent in six patients) and two UTR (singleton) SNPs. Notably, of the nine children who carried these exonic variants, all but one experienced ototoxicity following cisplatin treatment, and only two of the affected subjects also carried the risk allele at the index *ACYP2* SNP rs1872328, suggesting that additional variants at this locus might independently contribute to cisplatin-related hearing loss.

Determining the genetic basis for cisplatin-induced ototoxicity has been a formidable challenge. For example, a recent pharmacogenomic study focusing on genes involved in drug metabolism and transport identified variants in *TPMT* and *COMT* that were highly associated with hearing loss in children exposed to cisplatin, with genotype at these variants predicting 92.9% of the patients at risk[16]. Although some of these associations were replicated in subsequent validation by the same group[17], we and others have been unable to link variation in *TPMT* and *COMT* to platinum-induced ototoxicity[18,19]. These discrepancies can be attributed to differences in patient populations, platinum-containing therapies and methods of audiometric monitoring[20,21]. In particular, the use of non-concordant ototoxicity grading scales and audiometric testing performed many years after therapy can introduce unintended bias[22,23]. To mitigate the effects of these confounding variables, we elected to focus on patients treated on frontline clinical trials with systemic and well-controlled cisplatin therapy and regular and prospective audiometric monitoring. Our genome-wide approach of examining ~1.7 million SNPs in an unbiased fashion yielded a single susceptibility locus at 2p16.2 within the *ACYP2* gene at the genome-wide significance level. Three additional SNPs at this locus also showed independent associations, suggesting

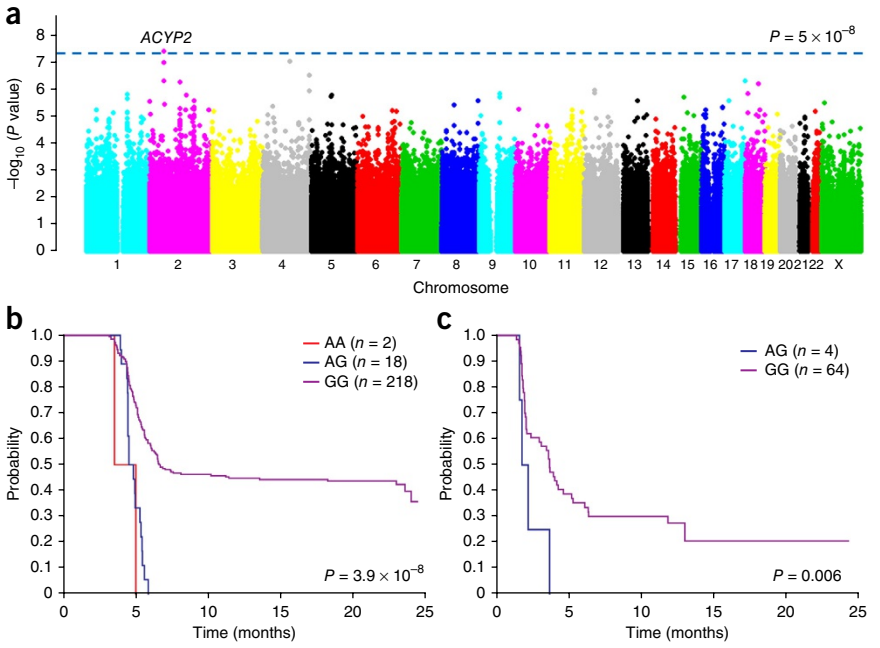


**Figure 1** Genome-wide association results of cisplatin-induced ototoxicity. (**a**) The association of SNP genotype and ototoxicity was evaluated using the Cox regression model for 1,716,999 SNPs in the discovery GWAS of 238 children with brain tumors uniformly treated with cisplatin-containing therapy. *P* values ($-\log_{10}$ *P* values; *y* axis) are plotted against the respective chromosomal position of each SNP (*x* axis). The gene symbol is indicated for the *ACYP2* locus (2p16.2) achieving the genome-wide significance threshold ($P = 5 \times 10^{-8}$; dashed blue line). (**b**,**c**) The relationships between genotype at *ACYP2* SNP rs1872328 and ototoxicity in the discovery GWAS series (SJMB96 and SJMB03 cohorts) (**b**) and the replication series (SJYC07 cohort) (**c**). *P* value was determined by two-sided time-dependent regression models (Online Methods).

the possibility of multiple variants within this genomic region collectively influencing the risk of ototoxicity. The further overrepresentation of the risk variant at *ACYP2* SNP rs1872328 in cases with early and severe hearing loss was validated in 68 children treated with identical doses of cisplatin and before any exposure to cranial irradiation, thus indicating a platinum-based susceptibility. Although all carriers of the *ACYP2* risk variant developed hearing loss rapidly, this variant only explained a relatively small proportion of the observed ototoxicity. In the combined discovery and replication cohorts, the specificity of ototoxicity prediction on the basis of rs1872328 genotype was 100% (of 112 patients who did not experience ototoxicity, 112 (100%) were correctly predicted by genotype (not carrying the risk allele)), with a sensitivity of 12.4% (of 194 patients who experienced ototoxicity, 24 (12.4%) carried the risk allele). The clinical usefulness of these findings should be examined in future trials, particularly in the context of potential clinical interventions for at-risk patients. GWAS with even greater sample sizes is also needed to characterize additional pharmacogenetic variants influencing cisplatin-related ototoxicity.

All of the ototoxicity-related SNPs within *ACYP2* identified in our study were intronic, and querying Encyclopedia of DNA Elements (ENCODE) and Epigenetics Roadmap data did not identify any obvious regulatory functions for these variants. However, other polymorphisms in the *ACYP2* gene have repeatedly been associated with severe neuropathy related to oxaliplatin[24,25], lending support for a broader relationship between *ACYP2* and toxicities of platinum agents. In fact, at the gene level, the expression of *ACYP2* was positively correlated with cisplatin cytotoxicity in lymphoblastoid cell lines *in vitro* ($P = 6.5 \times 10^{-5}$; **Supplementary Fig. 9**). Interestingly, genotype at the *ACYP2* SNP rs1872328 itself was not associated with cisplatin sensitivity *in vitro*, nor was it related to the expression of *ACYP2* and other genes within 300 kb of this index SNP (*TSPYL6*, *SPTBN1*, *PSME4* and *GPR75*) in these lymphoblastoid cells (data not shown). These observations raise the possibility that rs1872328 is a proxy marker for the causal functional variant that has yet to be identified. Alternatively, it is plausible that this genomic region encompassing rs1872328 functions as a *trans*-acting regulatory element that affects the transcription of genes much more distal to the index SNP. Our resequencing of the *ACYP2* gene identified additional rare exonic variants that were almost exclusively present in patients affected by hearing loss (**Supplementary Fig. 8**), adding evidence in support of this genomic region being a major risk locus. Further fine mapping in a larger cohort of patients will likely identify variants with independent associations with platinum-related ototoxicity.

*ACYP2* encodes an acylphosphatase that directly hydrolyzes phosphoenzyme intermediates of membrane pumps, with potential effects on $Ca^{2+}$ homeostasis[26]. Originally thought to be specific to muscle, *ACYP2* is also expressed in the cochlea[27] (**Supplementary Fig. 10**). The exact effects of *ACYP2* on $Ca^{2+}$ in the cochlea are unclear, but ATP-dependent $Ca^{2+}$ signaling is critical for hair cell development[28] and is directly implicated in hair cell damage[29]. These observations point to *ACYP2* as a plausible candidate gene underlying the association signal with ototoxicity at this locus, and future studies are warranted to characterize the molecular mechanisms by which it influences cisplatin-related cochlear cell death.

## METHODS

Methods and any associated references are available in the online version of the paper.

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*


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### AUTHOR CONTRIBUTIONS

J.J.Y. and C.F.S. supervised the research. A.G., G.W.R., A.B., M.C., U.B., S.G., T.H., M.F. and R.C. provided the study materials or patients. G.W.R., J.K.B., A.G., C.F.S., J.Y.-S.L., H.Z., T.T. and T.Y. collected and assembled the data. H.X., A.O.-T., J.H., J.Y.-S.L. and J.Z. analyzed and interpreted the data. All authors wrote and approved the manuscript.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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## ONLINE METHODS

**Patients and treatment.** A total of 238 children with newly diagnosed brain tumors enrolled in the St. Jude SJMB96 (ClinicalTrials.gov, NCT00003211; 1996–2003) and SJMB03 (NCT00085202; 2003–2012) protocols were included in the discovery GWAS, on the basis of the availability of germline DNA and audiology assessments (**Supplementary Fig. 2**). Patients with no cisplatin dose information ($n$ = 3) and/or with hearing loss at baseline ($n$ = 16) or before cisplatin treatment ($n$ = 1) were excluded. Tumor diagnosis included medulloblastoma ($n$ = 203), atypical teratoid rhabdoid tumor ($n$ = 13), pineoblastoma ($n$ = 14) and primitive neuroectodermal tumor ($n$ = 8) (**Supplementary Table 4**). Comparing study participants (included in the genetic analyses) with non-participants (treated on the clinical treatment protocols but not included in genetic analyses), we did not observe any statistically significant differences in demographic or clinical features (data not shown).

As described previously[30], SJMB96 was a frontline treatment protocol for newly diagnosed brain tumors with risk-adapted radiation and chemotherapy. Thus, patients with high-risk (metastatic and/or incompletely resected) medulloblastoma underwent craniospinal radiotherapy (M0–M1, 36Gy; M2–M3, 39.6 Gy) with a three-dimensional conformal boost to the tumor bed (total dose of 55.8 Gy) and, wherever appropriate, to local sites of metastasis (total dose of 50.4 Gy). Those with average-risk disease (M0 and the gross tumor totally resected or near totally resected) received 23.4 Gy of craniospinal radiotherapy with a boost to the tumor bed (total dose of 55.8 Gy). After a 6-week rest, all patients began four cycles of high-dose chemotherapy including cisplatin (75 mg/m$^2$ per cycle). The SJMB03 protocol used treatment regimens nearly identical to those of the SJMB96 protocol, except that (i) the clinical target volume margin for primary-site irradiation was 1 cm for SJMB03 and 2 cm for SJMB96 and (ii) all patients were offered amifostine as a prophylaxis for ototoxicity on the SJMB03 protocol, whereas patients on the SJMB96 protocol did not have the option to receive amifostine until 2000 (**Supplementary Fig. 1**)[12].

The replication cohort consisted of 68 young children with newly diagnosed brain tumor treated on the SJYC07 protocol (ClinicalTrials.gov, NCT00602667; **Supplementary Table 3**). In this protocol, patients were mostly younger than 3 years of age at diagnosis. Therapy was risk adapted into three treatment arms on the basis of diagnosis and clinical risk factors: a low-risk arm that consisted of chemotherapy only, an intermediate-risk arm that included focal radiation therapy after initial chemotherapy and a high-risk arm that consisted of chemotherapy only. Upfront craniospinal irradiation was avoided in the SJYC07 population, and cranial radiation, when administered, was limited to a defined margin around the tumor bed and given after chemotherapy. Although the protocol was inclusive of a multitude of diagnoses (medulloblastoma, supratentorial primitive neuroectodermal tumor, atypical teratoid rhabdoid tumor, high-grade glioma, choroid plexus carcinoma and ependymoma) and risk-adapted treatment regimens, all participants received identical induction chemotherapy regimens that only varied by the addition of vinblastine to the high-risk population. This induction regimen consisted of four cycles of therapy including cisplatin (75 mg/m$^2$ per cycle), followed by consolidation therapy whereby low-risk patients received two additional cycles of chemotherapy that included carboplatin, intermediate-risk patients received focal radiation therapy (54 Gy) to the tumor bed and high-risk patients received additional non-platinum-based chemotherapy.

This study was approved by the St. Jude Children's Research Hospital institutional review board, and informed consent was obtained from all patients, parents or legal guardians as appropriate.

**Hearing evaluation and ototoxicity.** For all patients enrolled on SJMB96, SJMB03 and SJYC07, ototoxicity was prospectively and regularly monitored according to the treatment protocols in a consistent fashion. Audiological evaluation for SJMB96 and SJMB03 was performed at enrollment (month 0), after radiotherapy (month 3), after each cycle of chemotherapy (months 4, 5, 6 and 7), every 2–3 months until 1 year after enrollment (months 9 and 12) and annually thereafter (months 24, 36 and so on). Audiological evaluation for SJYC07 was performed at enrollment (month 0), before the third cycle of chemotherapy (months 2–3), after the fourth cycle of chemotherapy and before consolidation therapy (month 5), after consolidation therapy (month 7), at the end of therapy (month 12) and annually thereafter (months 24, 36 and so on). Age- and developmentally appropriate audiometric testing was performed (for example, conventional audiometry, conditioned play, visual reinforcement audiometry or auditory brain stem response), and thresholds were measured at 0.25, 0.5, 1, 2, 3, 4, 6 and 8 kHz. Audiograms were evaluated using the Chang criteria[31].

Ototoxicity status was defined following our previously published procedures with slight modifications[18]. For children on the SJMB96 and SJMB03 protocols, cisplatin-related hearing loss assessment was based on audiology data obtained between 9 and 24 months after the initiation of therapy, and the audiology examination closest to 24 months and the worse grade for the two ears were used to determine ototoxicity. For children on the SJYC07 protocol in which cisplatin was administered over 4 months immediately after diagnosis, ototoxicity status was defined by the last audiology examination before 24 months. All ototoxicity grades were reviewed longitudinally to rule out temporary hearing loss (for example, otitis). Time to ototoxicity was defined as the lapse between the initiation of cisplatin therapy and the time when a nonzero Chang score was first recorded. Patients with Chang score >0 were classified as positive for ototoxicity, and events with Chang score ≥2a were considered as clinically relevant (when applicable).

**Genotyping and quality control.** Genotyping was performed using the Illumina HumanOmni2.5+HumanExome BeadChip. Genotype calls (coded as 0, 1 and 2 for AA, AB and BB genotypes) were determined using GenomeStudio software from Illumina. Samples for which the genotype was ascertained at <98% of SNPs on the array were deemed to have failed and were excluded from the analyses. SNP quality control procedures were performed on the basis of call rate (call rate > 95%) and MAF (MAF > 1%), and 1,716,999 of 2,602,667 SNPs were included in the GWAS (**Supplementary Fig. 4**).

**GWAS and replication.** In the discovery GWAS, ototoxicity was defined as Chang score > 0 and was modeled as a time-to-event variable to consider the onset of hearing loss relative to cisplatin therapy. The association of SNP genotype and ototoxicity was evaluated by the Cox regression model, with genetic ancestries (PC1–PC5 inferred by EIGENSTRAT[32]; **Supplementary Fig. 11**), craniospinal irradiation dose (<25 Gy or ≥25 Gy), treatment protocol (SJMB96 or SJMB03) and age at diagnosis as covariates. To ensure adequate correction for population stratification in the GWAS, we constructed a quantile-quantile plot and observed only minimal inflation at the upper tail of the distribution ($\lambda = 1.04$; **Supplementary Fig. 12**). Permutation was performed for genome-wide significant SNP(s) ($P < 5 \times 10^{-8}$) by randomly assigning the residuals from the regression model of ototoxicity and non-SNP variables[33]. The permutation $P$ value of a SNP is the fraction of the permutations for which this variant had a $P$ value less than or equal to that with the unpermutated data. The correlation of *ACYP2* SNP genotype (0, 1 and 2) and severity of ototoxicity (Chang scores 0, 1a–1b, 2a–2b and 3–4) was also evaluated using an ordinal regression approach.

*ACYP2* SNPs rs1872328 and rs7604464 were then tested in the replication study of 68 children from the SJYC07 protocol, for which we adopted the Fine and Gray regression model[34] to accommodate the relatively common progressive disease in this cohort as competing events (**Supplementary Note**).

Association between *ACYP2* gene expression and cisplatin $IC_{50}$ (concentration of cisplatin required for 50% inhibition of cell viability)[35] was assessed by a linear regression model in HapMap CEU (Utah residents of Northern and Western European ancestry) lymphoblastoid cell lines (Gene Expression Omnibus (GEO), GSE11582)[36].

R 3.0 statistical software was used for all analyses unless indicated otherwise. Statistical tests were two-sided and chosen as appropriate according to the phenotype distribution.

***ACYP2* resequencing.** Sanger sequencing was performed to identify additional variants in the exonic region of *ACYP2* in 257 patients included in the discovery and replication cohorts with sufficient germline genomic DNA. First, exons 1, 2, 3 and 4 were amplified by PCR (primer sequences are listed in **Supplementary Table 5**), and Sanger sequencing was then performed. Sequence analysis and variant calling were performed directly from chromatograms using CLC Genomics Workbench version 4.

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# Corrigendum: Common variants in *ACYP2* influence susceptibility to cisplatin-induced hearing loss

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