Published in final edited form as:

Nat Genet. 2015 March ; 47(3): 263–266. doi:10.1038/ng.3217.

Common Variants in *ACYP2* Influence Susceptibility to Cisplatin-induced Hearing Loss

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Abstract

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

Competing financial interests: The authors declare no competing financial interests

Taking a genome-wide association study approach, we identified inherited genetic variations in *ACYP2* associated with cisplatin 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. *ACYP2* risk variant strongly predisposed patients to precipitous hearing loss and was related to ototoxicity severity. These results point to novel biology of ototoxic effects of platinum agents.

Cisplatin is one of the most widely used anti-cancer agents, with well documented efficacy against a variety of solid tumors in children and adults¹⁻³. However, platinating agents are also known for debilitating adverse effects. Particularly in children, cisplatin-related hearing loss negatively impacts quality of life and severely impedes language development with irreversible long-term effects⁴. With cumulative cisplatin dosages at or above 400 mg/m², the auditory impairment is typically bilateral and highly prevalent with up to 70% of children suffering severe hearing loss necessitating hearing aids⁵. The proposed mechanism of ototoxicity is by the release and generation of both pro-apoptotic factors and free radicals within the sensory outer hair cells of the cochlea upon exposure to cisplatin⁴. While cisplatin is the most ototoxic, this adverse effect is not completely spared by the use of other platinum agents (e.g., carboplatin^{6,7}) and substitution is rarely performed when cisplatin is indicated due to concerns of inferior efficacy and/or prolonged myelosuppression from equivalent doses of carboplatin⁸.

Younger age and concurrent craniospinal irradiation have been reported to increase the risk of cisplatin 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 due to non-uniform patient populations, heterogeneous and non-protocol-based platinum therapies, and/or inadequate and inconsistent audiometric monitoring¹⁴. 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 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 single nucleotide polymorphisms (SNPs) associated with cisplatin 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 hereafter, Supplementary Figure 1 and 2), for whom hearing loss was prospectively monitored with a pre-defined schedule¹⁵. Ototoxicity primarily occurred between 1-6 months from start of cisplatin therapy (Supplementary Figure 3). Sixty-one percent of the patients developed detectable ototoxicity (Chang grade > 0) and 37% experienced clinically relevant ototoxicity (Chang grade ≥ 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 compared to the earlier SJMB96 treatment regimen, plausibly due to the reduced target volume of craniospinal irradiation and/or the use of amifostine. Gender, genetic ancestry, cumulative cisplatin dosage, or tumor location did not significantly influence ototoxicity (Table 1).

As quality control prior to GWAS, we first removed variants that were poorly genotyped (call rate < 98%) or rare (minor allele frequency < 1%). The final GWAS dataset included genotype at 1,716,999 variants in 238 children treated with cisplatin chemotherapy (Online Methods, Supplementary Figures 2 and 4). Treating hearing loss as a time-dependent variable, we compared the frequency and onset of hearing loss (Chang grade > 0) between 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 [95% CI]: 2.63-7.69, Figure 1a). Subsequent permutation test confirmed that the association at rs1872328 was beyond what would be expected by chance (permutation $P = 2 \times 10^{-6}$). No other genome-wide significant loci were observed. A second SNP (rs7604464, $P = 1 \times 10^{-7}$, HR = 3.81 [2.33-6.24]) in *ACYP2* also 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 Figure 5). Conditioning on rs1872328, 3 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 of the 20 patients (100%) carrying the A allele at rs1872328 developed ototoxicity, regardless of whether a patient was heterozygous or homozygous for the risk allele. In contrast, ototoxicity was noted in 57.3% children who do not carry this risk allele. More importantly, hearing loss in children with AA or AG genotype occurred in a particularly precipitous fashion, compared to those with GG genotype (Figure 1b). While 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 Figure 6). A strong correlation was also noted between rs1872328 genotype and the severity of ototoxicity ($P = 0.0005$), with the risk allele frequency increasing gradually with clinical grade of ototoxicity. The association at the *ACYP2* SNP was consistent regardless of the irradiation dose and across clinical protocols (Supplementary Figure 6).

To validate the association at the *ACYP2* locus, we genotyped rs1872328 and rs7604464 in young children (< 4 years old) with brain tumors treated on the St. Jude YC07 protocol (SJYC07) (Supplementary Figure 7 and Supplementary Table 3). Compared to the discovery GWAS cohort, patients on SJYC07 received the same cisplatin regimen but reduced and delayed irradiation in consideration of the young age. Of 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 heterozygous genotype of rs1872328 (no AA genotype was observed), all of whom developed ototoxicity (Figure 1c). Similar to the results in the discovery GWAS, children with the A allele suffered rapid hearing impairment, compared to those who do not carry the risk allele.

To further identify ototoxicity-related variants at the *ACYP2* locus, we resequenced exonic region of *ACYP2* in 257 children included in the discovery and validation cohorts (Supplementary Figure 8). A total of four variants were observed in nine patients: one missense (singleton), one silent (recurrent in 6 patients), and two UTR SNPs (singleton). Remarkably, of 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, pointing to additional variants at this locus independently contributing 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* highly associated with hearing loss in children exposed to cisplatin, with genotype at these variants predicting 92.9% of patients at-risk¹⁶. While some of these associations were replicated in subsequent validation by the same group¹⁷, we and others have been unable to link *TPMT* and *COMT* to platinum 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 the possibility of multiple variants within this genomic region collectively influencing the risk of ototoxicity. The further over-representation 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. While all carriers of the *ACYP2* risk variant developed hearing loss rapidly, it only explained a relatively small proportion of the observed ototoxicity. In the combined discovery and replication cohorts, the specificity of ototoxicity prediction based on rs1872328 genotype is 100% (i.e., 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% (i.e., of 194 patients who experienced ototoxicity, 24 [12.4%] carried the risk allele). The clinical utility of these findings should be examined in future trials, particularly in the context of potential clinical interventions for at-risk patients. GWAS of even greater sample sizes is also needed to characterize additional pharmacogenetic variants of cisplatin ototoxicity.

All of the ototoxicity-related SNPs within *ACYP2* identified in our study are intronic and query of the Encyclopedia of DNA Elements (ENCODE) and Epigenetics RoadMap data

did not reveal any obvious regulatory functions of these variants. However, other polymorphisms in the *ACYP2* gene have been repeatedly 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 Figure 9). Interestingly, the genotype at the *ACYP2* SNP rs1872328 itself was not associated with cisplatin sensitivity *in vitro*, and nor was it related to the expression of *ACYP2* and other genes 300 Kb within this index SNP (i.e., *TSPYL6*, *SPTBN1*, *PSME4*, and *GPR75*) in these lymphoblastoid cells (data not shown). These observations raised the possibility that rs1872328 is a proxy marker for the causal functional variant that is yet to be identified. Alternatively, it is plausible that this genomic region encompassing rs1872328 functions as a trans-acting regulatory element that affects transcription of genes much more distal to the index SNP. Our resequencing of *ACYP2* gene revealed additional rare exonic variants that were almost exclusively present in patients affected by hearing loss (Supplementary Figure 8), adding evidence in support of this genomic region as major risk locus. Further fine-mapping in a larger cohort of patients will likely to identify variants with independent association with platinum ototoxicity.

ACYP2 encodes an acylphosphatase that directly hydrolyzes phosphoenzyme intermediate of membrane pumps, with potential effects on Ca^{2+} homeostasis²⁶. Originally thought to be muscle-specific, *ACYP2* is also expressed in the cochlea²⁷ (Supplementary Figure 10). The exact effects of *ACYP2* on Ca^{2+} in the cochlea is unclear, but ATP-dependent Ca^{2+} signaling is critical for hair cell development²⁸ and directly implicated in hair cell damage²⁹. While these observations point to *ACYP2* as a plausible candidate gene underlying the association signal with ototoxicity at this locus, future studies are warranted to characterize the molecular mechanisms by which it influences cisplatin-related cochlea cell death.

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, based on the availability of germline DNA and audiology assessments (Supplementary Figure 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 neuroectodermall 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 significant differences in demographic or clinical features (data not shown).

As described previously³⁰, 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 (M0–1, 36Gy; M2–3, 39.6Gy) with a three-dimensional conformal boost to the

tumor bed (total dose: 55.8Gy) and, wherever appropriate, to local sites of metastasis (total dose: 50.4Gy). Those with average-risk disease (M0 and gross totally resected/near totally resected) received 23.4Gy craniospinal radiotherapy, with boost to the tumor bed (total dose: 55.8Gy). After a 6-week rest, all patients began four cycles of high-dose chemotherapy including cisplatin (75 mg/m² per cycle). The SJMB03 protocol utilized treatment regimens nearly identical to those of the SJMB96 protocol, except that (i) clinical target volume margin for primary site irradiation was 1cm for SJMB03 and 2cm for SJMB96 and (ii) all patients were offered amifostine as a prophylaxis for ototoxicity on SJMB03, whereas patients on the SJMB96 protocol did not have the option to receive amifostine until 2000¹² (Supplementary Figure 1).

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 based on 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 inclusive of a multitude of diagnoses (medulloblastoma, supratentorial primitive neuroectodermal tumor, atypical teratoid rhabdoid tumor, high grade glioma, choroid plexus carcinoma, or ependymoma) and risk-adapted treatment regimens, all participants received identical induction chemotherapy regimens which only varied by the addition of vinblastine to the high risk population. This induction regimen consisted of 4 cycles of therapy including cisplatin (75 mg/m² per cycle), followed by consolidation therapy whereby low risk patients received additional 2 cycles of chemotherapy which includes carboplatin, intermediate risk patient received focal radiation therapy (54Gy) to the tumoral 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 per 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 (month 4, 5, 6, 7), every 2-3 months until 1 year from enrollment (month 9 and month 12) and thereafter annually (month 24, 36, and so on). Audiological evaluation for SJYC07 was performed at enrollment (month 0), prior to the third cycle of chemotherapy (month 2-3), after the fourth cycle of chemotherapy and prior to consolidation (month 5), after consolidation therapy (month 7), at end of therapy (12 months) and thereafter annually (month 24, 36, and so on). Age- and developmentally- appropriate audiometric testing was performed (e.g., 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 criteria³¹.

Ototoxicity status was defined following our previously published procedures with slight modifications¹⁸. For children on the SJMB96 and SJMB03 protocols, cisplatin-related hearing loss assessment was based on audiology data between 9 and 24 months from therapy initiation, and the audiology examination closest to 24 months and the worse grade of two ears were used to determine ototoxicity. For children on SJYC07 protocol in which cisplatin was administered over 4 months immediately after diagnosis, ototoxicity status was thus defined by the last audiology examination prior to 24 months. All ototoxicity grades were reviewed longitudinally to rule out temporary hearing loss (e.g., otitis). Time to ototoxicity was defined as the lapse between cisplatin therapy initiation and the time when a non-zero Chang grade was first recorded. Patients with Chang grade > 0 were classified as positive for ototoxicity, and events with Chang grade 2a were considered as clinically relevant (when applicable).

Genotyping and quality control

Genotyping was performed by using the Illumina HumanOmni2.5+HumanExome BeadChip (Illumina). Genotype calls (coded as 0, 1, and 2 for AA, AB, and BB genotypes) were determined using the GenomeStudio Software from Illumina. Samples for which 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 minor allele frequency (MAF > 1%), and 1,716,999 of 2,602,667 SNPs were included in the GWAS (Supplementary Figure 4).

GWAS and replication

In the discovery GWAS, ototoxicity was defined as Chang grade > 0 and was modeled as a time-to-event variable to consider the onset of hearing loss relative to cisplatin therapy. Association of SNP genotype and ototoxicity was evaluated by the Cox regression model, with genetic ancestries (PC1-5 inferred by EIGENSTRAT³², Supplementary Figure 11), craniospinal irradiation dose (< 25Gy or ≥ 25Gy), 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 (Q-Q) plot and there was only minimal inflation at the upper tail of the distribution ($\lambda = 1.04$, Supplementary Figure 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 variables³³. 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 unpermuted data. The correlation of *ACYP2* SNP genotype (0, 1, and 2) and severity of ototoxicity (Chang grade 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

model³⁴ to accommodate the relatively common progressive disease in this cohort as competing events.

Association between *ACYP2* gene expression and cisplatin IC₅₀³⁵ was assessed by a linear regression model in HapMap CEU lymphoblastoid cell lines (GSE11582³⁶).

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 the *ACYP2* gene 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 (primers are listed in Supplementary Table 5), followed by Sanger sequencing. Sequence analysis and variant calling was performed directly from chromatograms using the CLC Genomics Workbench version 4.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Stacy Throm, PhD, for coordinating patient samples, Sarah Hughes for curating the ototoxicity data, all patients and their parents who participated in the St. Jude protocols included in this study, Grace Koh from Department of Paediatrics, Yong Loo Lin School of Medicine, National University of Singapore for her assistance in genome-wide genotyping, and Leslie Robison PhD and Kirsten Ness PhD at Department of Epidemiology and Cancer Control at St. Jude for insightful discussions. This work was supported by National Institutes of Health (P30CA21765-34 and U01GM92666) and American Lebanese Syrian Associated Charities (ALSAC).

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Figure 1. Genome-wide association results of cisplatin-induced ototoxicity

(a) Association of SNP genotype and ototoxicity was evaluated using the Cox regression model for 1,716,999 SNPs in the discovery GWAS of 268 children with brain tumors uniformly treated with cisplatin-containing therapy. P values ($-\log_{10} P$, y axis) were plotted against respective chromosomal position of each SNP (x axis). Gene symbol was indicated for ACYP2 locus (2p16.2) achieving genome-wide significance threshold ($P < 5 \times 10^{-8}$, dashed blue line). (b) and (c) Relationships between genotype at ACYP2 SNP rs1872328 and ototoxicity in the discovery GWAS series (SJMB96 and SJMB03 cohort, b) and replication series (SJYC07 cohort, c), respectively. P value was determined by two-sided time-dependent regression models as described in Online Methods.

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

Features	Ototoxicity status		<i>P</i> value(time to event)
	Chang = 0 (N = 93)	Chang > 0 (N = 145)	
	Mean ± std		
Age at diagnosis (years old)	10.0 ± 4.3	8.5 ± 3.8	0.014
Cisplatin Cumulative Dosage (mg/m ²)	288.7 ± 36.1	286.5 ± 34.8	0.3
	Number of Patients (%)		
Gender			
female	36 (40%)	54 (60%)	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%)	120 (58%)	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)	0.2
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* < 0.05 is shown in bold. Genetic ancestry was determined by genome-wide SNP genotype using EIGENSTRAT.