

Genetic variants in *TPMT* and *COMT* are associated with hearing loss in children receiving cisplatin chemotherapy

Colin J D Ross^{1,2,11}, Hagit Katzov-Eckert^{1,2,11}, Marie-Pierre Dubé³, Beth Brooks⁴, S Rod Rassekh⁵, Amina Barhdadi³, Yassamin Feroz-Zada³, Henk Visscher^{1,2}, Andrew M K Brown^{3,6}, Michael J Rieder⁷, Paul C Rogers⁵, Michael S Phillips^{3,6}, Bruce C Carleton^{2,8,9} Michael R Hayden^{1,2} & the CPNDS Consortium¹⁰

Cisplatin is a widely used and effective chemotherapeutic agent, although its use is restricted by the high incidence of irreversible ototoxicity associated with it¹. In children, cisplatin ototoxicity is a serious and pervasive problem, affecting more than 60% of those receiving cisplatin^{2–5} and compromising language and cognitive development. Candidate gene studies have previously reported associations of cisplatin ototoxicity with genetic variants in the genes encoding glutathione S-transferases and megalin^{6–8}. We report association analyses for 220 drug-metabolism genes in genetic susceptibility to cisplatin-induced hearing loss in children. We genotyped 1,949 SNPs in these candidate genes in an initial cohort of 54 children treated in pediatric oncology units, with replication in a second cohort of 112 children recruited through a national surveillance network for adverse drug reactions in Canada. We identified genetic variants in *TPMT* (rs12201199, *P* value = 0.00022, OR = 17.0, 95% CI 2.3–125.9) and *COMT* (rs9332377, *P* value = 0.00018, OR = 5.5, 95% CI 1.9–15.9) associated with cisplatin-induced hearing loss in children.

Cisplatin has been described as one of the most ototoxic drugs in clinical use, causing serious, permanent, bilateral hearing loss in 10–25% of adults receiving the drug, 50% of individuals receiving high doses (>400 mg/m²) and 41–61% of children^{2–5,9}. In children, the impact of hearing loss is most profound because even mild losses of hearing considerably increase a child's risk of learning difficulties and social-emotional problems^{4,10}. Cisplatin ototoxicity frequently leads to dose reduction and premature termination of cisplatin treatment, which may affect overall survival rates. There is substantial inter-individual variation in ototoxicity in individuals receiving similar doses of cisplatin; this has led to the hypothesis that some individuals

have polymorphisms in genes encoding drug-metabolizing enzymes that render them especially susceptible to cisplatin ototoxicity¹¹.

A discovery cohort of 54 children treated in pediatric oncology units who received cisplatin therapy was recruited from the BC Children's Hospital in Vancouver, British Columbia, Canada (Table 1). Children who suffered serious cisplatin-induced ototoxicity (*n* = 33) were defined by the development of grade 2–4 hearing impairment following cisplatin therapy using Cancer Therapy Evaluation Program, Common Terminology Criteria for Adverse Events (CTCAE) criteria, showing a hearing loss of >25 dB at frequencies of 4–8 kHz. In this cohort, 22 (40%) children on cisplatin treatment did not experience any significant hearing loss (CTCAE grade 0). To better differentiate between cisplatin ototoxicity and normal hearing, children with hearing loss defined as grade 1 (*n* = 4) were removed from the initial analysis.

A second, independent replication cohort of 112 children treated in pediatric oncology units who received cisplatin chemotherapy was recruited from pediatric oncology units across Canada (Table 1). In this cohort, 73 (66%) children suffered serious (defined as grade 2–4) cisplatin-induced ototoxicity. In the replication and combined cohorts, male gender was moderately associated with ototoxicity (67% compared to 50% in females, *P* value = 0.042 overall) and fewer children with germ-cell tumors developed ototoxicity (6.6% versus 26.8%, *P* value = 0.0006 overall), although these differences did not show a similar trend in the discovery cohort.

Study participants' DNA samples were genotyped for 1,949 SNPs using an Illumina GoldenGate assay designed to capture the genetic variation in 220 key genes involved in the absorption, distribution, metabolism and elimination (ADME) of medications and their metabolites (Supplementary Table 1). We used a tiered analysis strategy to identify variants that were highly associated with cisplatin ototoxicity in the initial discovery cohort (*P* < 0.01) and that remained highly associated in a second replication cohort (*P* < 0.01)¹². The key

¹Department of Medical Genetics, University of British Columbia, Centre for Molecular Medicine and Therapeutics, Vancouver, British Columbia, Canada.

²Child and Family Research Institute, Children's and Women's Health Research Centre of British Columbia, Vancouver, British Columbia, Canada. ³Montreal Heart Institute and Université de Montréal, Montreal, Quebec, Canada. ⁴Audiology and Speech Pathology Department and ⁵Department of Pediatrics, Division of Pediatric Hematology/Oncology/Bone and Marrow Transplant, British Columbia Children's Hospital, Vancouver, British Columbia, Canada. ⁶Montreal Heart Institute and Genome Québec Pharmacogenomics Centre, Montreal, Quebec, Canada. ⁷Department of Paediatrics, Children's Hospital at the London Health Sciences Centre, London, UK. ⁸Faculty of Pharmaceutical Sciences and ⁹Department of Paediatrics, Pharmaceutical Outcomes Programme, University of British Columbia, Vancouver, British Columbia, Canada. ¹⁰A full list of members is provided in the **Supplementary Note**. ¹¹These authors contributed equally to this work. Correspondence should be addressed to M.R.H. (mrh@cmm.ubc.ca).

Received 15 May; accepted 1 October; published online 8 November 2009; doi:10.1038/ng.478

Table 1 Subject demographics

	Discovery (<i>n</i> = 53)			Replication (<i>n</i> = 109)			Combined (<i>n</i> = 162)		
	Ototox. (<i>n</i> = 33)	Controls (<i>n</i> = 20)	<i>P</i> value	Ototox. (<i>n</i> = 73)	Controls (<i>n</i> = 36)	<i>P</i> value	Ototox. (<i>n</i> = 106)	Controls (<i>n</i> = 56)	<i>P</i> value
Age, y (median (min, max))	5.0 (0, 16)	9.0 (0, 16)	0.213	6 (0, 16)	9.5 (1, 19)	0.187	6 (0, 16)	9 (0, 19)	0.086
Dose, cumulative mg/m ² (median (min, max))	360 (180, 630)	360 (180, 720)	0.854	400 (120, 720)	410 (100, 700)	0.469	400 (120, 720)	400 (100, 720)	0.655
Treatment duration, wk (median (min, max))	4.0 (1, 11)	4.5 (1, 9)	0.365	5.0 (1, 12)	4.0 (1, 11)	0.673	5.0 (1,12)	4.0 (1, 11)	0.890
Gender (male <i>n</i> , (%))	22 (66.7%)	14 (70.0%)	1.000	49 (67.1%)	14 (38.9%)	0.0072 ^a	71 (67.0%)	28 (50.0%)	0.0425 ^a
Concomitant medication (<i>n</i> , (%))									
Tobramycin	0	0		18 (24.66%)	10 (27.8%)	0.267	18 (17.0%)	10 (17.9%)	0.275
Vancomycin	1 (3.03%)	1 (5%)	1.000	12 (16.44%)	5 (13.9%)	1.000	13 (12.3%)	6 (10.7%)	0.764
Vincristine	2 (6.06%)	0		7 (9.59%)	0	0.176	9 (8.5%)	0	0.055
Gentamicin	0	0		10 (13.70%)	3 (8.3%)	1.000	10 (9.4%)	3 (5.4%)	1.000
Tumor type (<i>n</i> , (%))									
Brain tumor	8 (24.2%)	2 (10.0%)	0.286	17 (23.3%)	6 (16.7%)	0.467	25 (23.6%)	8 (14.3%)	0.219
Endodermal sinus tumor of thymus	0	0		0	1 (2.8%)	0.330	0	1 (1.8%)	0.346
Germ cell tumor	2 (6.1%)	3 (15.0%)	0.354	5 (6.9%)	12 (33.3%)	0.0012 ^a	7 (6.6%)	15 (26.8%)	0.0006 ^a
Hepatoblastoma	7 (21.2%)	2 (10.0%)	0.456	15 (20.6%)	3 (8.3%)	0.169	22 (20.8%)	5 (8.9%)	0.075
Lymphoma	0	0		0	1 (2.8%)	0.330	0	1 (1.8%)	0.346
Nasopharyngeal carcinoma	0	0		1 (1.4%)	0	0.121	1 (0.9%)	0	1.000
Neuroblastoma	8 (24.2%)	4 (20.0%)	1.000	18 (24.7%)	5 (13.9%)	0.223	26 (24.5%)	9 (16.1%)	0.233
Osteosarcoma	7 (21.2%)	8 (40.0%)	0.209	17 (23.3%)	8 (22.2%)	1.000	24 (22.6%)	16 (28.6%)	0.446
Sarcoma	1 (3.0%)	1 (5.0%)	1.000	0	0		1 (0.9%)	1 (1.8%)	1.000
Cranial irradiation (<i>n</i> , (%))	7 (21.2%)	2 (10.0%)	0.456	16 (21.9%)	5 (13.9%)	0.440	23 (21.7%)	7 (12.5%)	0.202

For age, dose and treatment duration, the Wilcoxon-Mann-Whitney test with normal approximation was used. For gender, medication, tumor type and cranial irradiation, the Fisher exact test was used. Ototox., individuals with ototoxicity.

^aStatistically significant at 0.05 type I error rate.

advantages of this approach are increased power in the discovery cohort because the subjects were ascertained from a single site, yielding a more homogeneous sample set less subject to biases, and the fact that the broadly ascertained replication cohort ensured generalizability of clinically significant findings and minimized the likelihood of false positives. We also combined the cohorts to demonstrate the overall level of significance to show that these variants remained highly significant after correction for multiple testing. We identified two SNPs in the genes encoding thiopurine S-methyltransferase (*TPMT*) and catechol O-methyltransferase (*COMT*) that were highly associated with cisplatin-induced deafness in the discovery cohort and that were replicated in the second cohort (**Table 2**).

TPMT rs12201199 showed similar effect sizes in both the discovery and replication cohorts. The risk allele was present in 9 (27.3%) and 16 (21.9%) of the individuals with cisplatin ototoxicity in the discovery and replication cohorts, whereas it was not present in controls without cisplatin ototoxicity in the discovery cohort and was present in only 1 (2.4%) control in the replication cohort, conferring odds ratios of 15.9 (95% CI 0.87–290.0) and 9.82 (95% CI 1.25–77.37), respectively. The *TPMT* variants remained significant after Bonferroni correction for multiple testing in a combined analysis (Fisher exact allelic test $P = 2.2 \times 10^{-4}$, Bonferroni-corrected $P = 0.032$). The *COMT* A allele of rs4646316 was present in 10 (50.0%) and 21 (58.3%) of the controls in the discovery and replication cohorts, respectively, but in only 10 (33.3%) and 21 (29.2%) of the individuals with cisplatin ototoxicity in the two cohorts. The protective AA genotype conferred odds ratios of 23.8 (95% CI 1.2–457.5) and 4.2 (95% CI 0.4–48.3) in the discovery and replication cohorts, respectively.

Because the polymorphisms in *TPMT* and *COMT* could be in linkage disequilibrium (LD) with other causal variants in the regions, in follow-up analyses, we sequenced *TPMT* and *COMT* genes in the children with cisplatin ototoxicity (*n* = 106) and controls without cisplatin ototoxicity (*n* = 56). DNA sequencing of *TPMT* revealed two previously described loss-of-function variants that eliminate normal

TPMT enzyme activity, present in 17 of 25 (68%) of the individuals with cisplatin ototoxicity having the rs12201199 variant. The loss-of-function variant rs1142345 (*TPMT**3C, Tyr240Cys) was present in all 17 of these individuals, and rs1800460 (*TPMT**3B, Ala154Thr) was also present in 15 of these individuals and none of the controls (**Table 2**). Individuals who carry both the rs1142345 (*TPMT**3C) and rs1800460 (*TPMT**3B) variants are defined as carriers of the *TPMT**3A haplotype. These findings suggest that the association of cisplatin ototoxicity with rs12201199 is likely due to linkage with rs1142345 and rs1800460. At least 23 variant alleles of *TPMT* have now been reported¹³, and DNA sequencing of the entire coding region and intron boundaries of the *TPMT* gene did not reveal other potentially causal variants in these study participants.

DNA sequencing of *COMT* revealed an additional variant associated with cisplatin ototoxicity, rs9332377 in a 7.5-kb haplotype block with rs4646316 (**Supplementary Fig. 1**). In a combined analysis, the rs9332377 variant remained significant after correction for multiple testing (Fisher exact allelic test $P = 0.000182$, Bonferroni-corrected $P = 0.026$) (**Table 2**). The haplotype of rs9332377 A and rs4646316 G carried a low-activity synonymous *COMT* variant, rs4818, that showed a trend toward an association with cisplatin ototoxicity (Fisher exact allelic test $P = 0.014$). The synonymous rs4818 variant confers an 11- to 18-fold reduction in *COMT* activity and protein levels through alterations in mRNA secondary structure^{14,15}.

In this study, we decided *a priori* to compare individuals with no hearing loss to individuals with grade 2–4 ototoxicity because in standardized cisplatin chemotherapy protocols, clinical intervention occurs at grade 2 ototoxicity, at which point the dose of cisplatin is either reduced or discontinued (**Supplementary Table 2**). Thus, grade 2 individuals might have gone on to develop grade 3 or 4 toxicity if cisplatin therapy had not been discontinued. Grade 1 individuals were excluded to better discriminate between no hearing loss and serious hearing loss. Looking back at the individuals with grade 1 ototoxicity in both cohorts, three of the four carried *TPMT* or *COMT* ototoxicity

Table 2 Genetic variants associated with cisplatin-induced hearing loss

SNP	Genotype/ allele	Discovery (n=53)				Replication (n = 109)				Combined (n = 162)				Adjusted P value ^b	
		Ototox. (n = 33)	Controls (n = 20)	OR (95% CI)	P value ^a	Ototox. (n = 73)	Controls (n = 36)	OR (95% CI)	P value ^a	Ototox. (n = 106)	Controls (n = 56)	OR (95% CI)	P value ^a		
TPMT															
rs12201199	A/A	1	0	2.51 (0.1, 65.00)	–	2	0	3.09 (0.14, 66.17)	0.528	3	0	4.77 (0.24, 94.11)	0.277	1.000	
	A/T	8	0	14.22 (0.77, 261.62)	0.017	14	1	8.60 (1.08, 68.26)	0.018	22	1	14.94 (1.96, 114.09)	0.00061	0.086	
	A/_	9	0	15.90 (0.87, 290.02)	0.0097	16	1	9.82 (1.25, 77.37)	0.010	25	1	16.98 (2.23, 128.99)	0.000181^c	0.0231^c	
	T/T	24	20	1		57	35	1		81	55	1			
	A	10	0	14.29 (0.81, 251.74)	0.0097	18	1	9.98 (1.31, 76.36)	0.0071	28	1	16.89 (2.27, 125.88)	0.00022^c	0.0318^c	
rs1142345	G/G	0	0	–	1	2	0	2.75 (0.13, 58.92)	0.543	2	0	3.10 (0.15, 65.79)	0.527	1.000	
	G/A	8	0	13.67 (0.74, 251.10)	0.019	7	1	3.83 (0.45, 32.39)	0.264	15	1	9.27 (1.19, 72.15)	0.011	0.812	
	G/_	8	0	13.67 (0.74, 251.10)	0.019	9	1	4.92 (0.60, 40.46)	0.160	17	1	10.51 (1.36, 81.17)	0.0068	0.633	
	A/A	25	20	1		64	35	1		89	55	1			
	G	8	0	11.03 (0.61, 197.64)	0.022	11	1	5.79 (0.73, 45.72)	0.044	19	1	10.93 (1.44, 82.74)	0.0017	0.221	
rs1800460	A/A	0	0	–	1	1	0	1.60 (0.06, 40.34)	1.000	1	0	1.82 (0.07, 45.45)	0.999	1.000	
	A/G	8	0	13.67 (0.74, 251.10)	0.019	6	0	6.94 (0.38, 126.78)	0.174	14	0	17.59 (1.03, 300.74)	0.0020	0.244	
	A/_	8	0	13.67 (0.74, 251.10)	0.019	7	0	8.01 (0.44, 144.31)	0.094	15	0	18.80 (1.10, 320.51)	0.0013	0.175	
	G/G	25	20	1		66	35	1		91	55	1			
	A	8	0	11.03 (0.61, 197.64)	0.022	8	0	8.12 (0.46, 143.37)	0.046	16	0	17.96 (1.07, 302.66)	0.00312	0.413	
COMT	G	58	40			138	70			196	110				
	rs4646316	G/G	20	10	21.48 (1.08, 426.70)	0.0093	51	15	6.80 (0.58, 80.28)	0.148	71	25	19.88 (2.33, 169.70)	0.00098	0.135
		G/A	13	5	27.00 (1.27, 575.95)	0.0075	21	19	2.21 (0.19, 26.38)	0.607	34	24	9.92 (1.14, 85.95)	0.022	0.959
		G/_	33	15	23.77 (1.24, 457.45)	0.0054	72	34	4.24 (0.37, 48.34)	0.253	105	49	15.00 (1.80, 125.29)	0.0026	0.321
		A/A	0	5	1		1	2	1		1	7	1		
G		53	25	2.45 (1.01, 5.91)	0.044	123	49	2.51 (1.29, 4.89)	0.0059	176	74	2.51 (1.48, 4.27)	0.00055	0.076	
rs9332377	A	13	15			23	23			36	38				
	A/A	1	0	2.71 (0.10, 70.65)	1.000	4	0	5.59 (0.29, 107.16)	0.293	5	0	7.65 (0.41, 141.32)	0.156	1.000	
	A/G	12	2	5.40 (1.06, 27.47)	0.052	14	2	4.71 (1.01, 21.95)	0.051	26	4	4.51 (1.48, 13.68)	0.0052	0.538	
	A/_	13	2	5.85 (1.16, 29.54)	0.028	18	2	5.56 (1.21, 25.49)	0.017	31	4	5.37 (1.79, 16.14)	0.00109	0.148	
	G/G	20	18	1		55	34	1		75	52	1			
A	14	2	5.12 (1.10, 23.85)	0.024	22	2	6.21 (1.42, 27.19)	0.0087	36	4	5.52 (1.91, 15.95)	0.000182^c	0.0261^c		
	G	52	38			124	70			176	108				

^aDetermined using Fisher exact test. ^bDetermined using Bonferroni-corrected P value. ^cP < 0.05 after Bonferroni multiple testing correction. Ototox., individuals with ototoxicity.

risk variants. In an analysis of all affected individuals including those with grade 1 ototoxicity ($n = 166$), the results for *TPMT* and *COMT* are made more significant when the grade 1 and grade 2–4 individuals are grouped together (*TPMT* rs12201199, $P = 6.2 \times 10^{-5}$, *COMT* rs9332377, $P = 1.1 \times 10^{-4}$) (Supplementary Table 3). Similarly, in an analysis of individuals with severe hearing loss (grade 3 and 4) versus controls with no ototoxicity, *TPMT* remains significant (Supplementary Table 4).

Using the Eigenstrat principal component analysis, we found that the majority of the study participants (85%) were of European ancestry^{16,17} (Supplementary Fig. 2). In a subgroup analysis of only individuals of European ancestry ($n = 143$), the associations became stronger for the variants associated with cisplatin ototoxicity (Supplementary Table 5).

A Kaplan-Meier graph of an increasing number of *TPMT* and *COMT* risk allele combinations showed an earlier onset ($P = 0.00009$) and a greater severity of cisplatin-induced hearing loss for individuals with more than one risk allele ($P = 0.0000027$) (Fig. 1). The occurrence of *TPMT* and *COMT* risk genotypes in affected individuals did not substantially overlap, and a combination of cisplatin-ototoxicity variants cumulatively accounted for 48.1% of the individuals with cisplatin ototoxicity (Table 3).

Individuals with cisplatin ototoxicity were more likely to be male (67.0%; $P = 0.042$) (Table 1). Regression analysis revealed that *TPMT* rs12201199 and *COMT* rs4646316 remained significant

after adjustment for age and gender in the combined cohorts ($P = 0.009$, $P = 0.0024$, respectively).

The baseline hearing values for all of the children in these pediatric oncology cohorts were normal (grade 0) before they received cisplatin. To investigate whether susceptibility to deafness in the absence of cisplatin is affected by these variants, we genotyped 192 children referred to the BC Children's Hospital for sensorineural hearing loss who had not been on cisplatin therapy. We confirmed that these variants were indeed not seen at higher frequency in children with general deafness compared to the general population, but are likely specific to cisplatin-induced ototoxicity.

The frequency of the *TPMT* tag SNP rs12201199 is 5% in Europeans, 1.7% in Asians and 51.7% in Africans¹⁸. The African population has a unique LD structure in which the tag SNP rs12201199 is not in LD with rs1142345 and rs1800460, demonstrating the greater genetic diversity in this population (Supplementary Fig. 1a). In African populations, the higher frequency of *TPMT* loss-of-function variants would be expected to increase the frequency of cisplatin ototoxicity. Indeed, African-American subjects are more likely than the general US population to develop cisplatin-induced nephrotoxicity¹⁹; however, the influence of ancestry on cisplatin-induced ototoxicity has not previously been reported.

Although the endogenous substrate of *TPMT* is currently unknown, *TPMT* can methylate and inactivate exogenous thiopurine compounds, such as the metabolites of azathioprine²⁰. Genotyping of

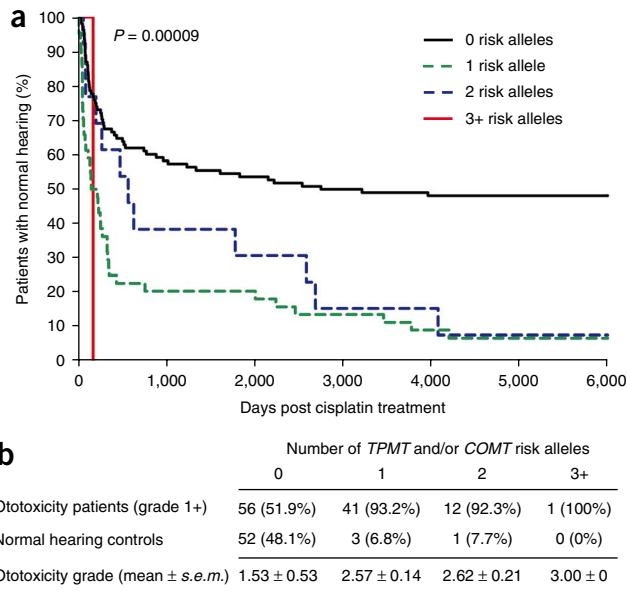


Figure 1 Cisplatin ototoxicity and number of risk alleles. (a) The Kaplan-Meier graph of cisplatin ototoxicity and number of *TPMT* and *COMT* risk alleles shows that an increasing number of *TPMT* rs12201199 and *COMT* rs9332377 risk alleles is associated with earlier onset of cisplatin-induced hearing loss ($P = 0.00009$). (b) An increasing number of these risk alleles is also associated with more severe cisplatin-induced hearing loss ($P = 0.000027$).

TPMT SNPs rs1142345 and rs1800460 is particularly important to identify individuals with an increased probability of adverse events, in particular bone marrow toxicity, if treated with standard doses of mercaptopurine or azathioprine^{21,22}. Cisplatin normally binds thiol-containing compounds and purines, especially guanine, and exerts its cytotoxic effect by forming intra- and interstrand DNA cross-links, causing cell death in rapidly dividing cells. One potential mechanism of decreased *TPMT* enzyme activity that causes cisplatin ototoxicity may function through reduced inactivation of cisplatin-purine compounds, thereby increasing the efficiency of cisplatin cross-linking and increasing cisplatin toxicity.

TPMT and *COMT* are methyltransferases dependent on the S-adenosylmethionine (SAM) methyl donor substrate in the methionine pathway^{23,24}. A putative mechanism for cisplatin ototoxicity through reduced *TPMT* and *COMT* activity could be mediated through increased levels of this substrate. In a recent study, administration of SAM to mice was not toxic, and administration of cisplatin alone resulted in only moderate toxicity, whereas administration of SAM and cisplatin together increased cisplatin toxicity substantially, by

3–6.2-fold, as monitored by renal dysfunction²⁵. These results suggest that cisplatin-induced ototoxicity could be related to increased levels of SAM through reduced *TPMT* or *COMT* activity. Recent studies have also shown that *LRTOMT2*, an enzyme with 60% similarity to *COMT* for 212 conserved amino acids including the substrate-binding region, appears to function as a catechol-O-methyltransferase and is essential for auditory function in mice and humans^{26,27}.

We did not identify significant associations with the previously reported associations with cisplatin ototoxicity for the megalin rs2075252 A allele (in our combined cohort, Fisher exact allelic P value = 0.55, OR = 1.2), the protective *GSTP1* rs1695 G-G genotype (Fisher exact P value = 0.61, OR = 0.71) or the presence of the *GSTM1* gene (Fisher exact P value = 0.51, OR = 0.78).

In this study, the strong associations of cisplatin-induced hearing loss with specific genetic variants were identified with a relatively small number of samples, likely due to the large effect size. The success of large-scale screens with relatively small numbers of affected individuals is limited to those situations in which there is a small number of relatively common adverse drug reaction (ADR) genetic risk factors, each with a large effect²⁸. For severe ADRs where the genetic effect is large, such as the cisplatin-induced ototoxicity reported here, carbamazepine-induced Stevens-Johnson syndrome, abacavir-induced hypersensitivity, statin-induced myopathy²⁹ and gefitinib-induced diarrhea, the number of affected individuals required to identify a highly significant association using a large, genome-wide scan is 10–100 people²⁸. This is supported by the retrospective genome-wide analysis of the abacavir-hypersensitivity reaction, where a marker in LD with the causal HLA-B*5701 variant was among the 10 most significant SNPs when as few as 15 hypersensitivity reaction cases were analyzed²⁸.

These findings suggest that it may be possible to identify individuals at higher risk of cisplatin ototoxicity based on genotype, which would improve counseling and treatment options. Alternative treatment options may include lower doses of cisplatin or treatment with carboplatin, which shows a nearly similar cure rate with reduced ototoxicity. Individuals at higher risk may also be selected for experimental otoprotectant studies. Validation of these findings in additional pediatric cohorts is required prior to considerations of clinical application. Investigation of these variants in adult populations is also necessary to understand the contribution of these variants to cisplatin ototoxicity in adults, where, for example, cisplatin is the drug of choice in the management of ovarian cancer. It is likely that other genetic factors are also involved, and after recruitment of a sufficient number of participants, a genome-wide association study will be a valuable next step to identify additional genetic factors that may be involved in cisplatin-induced ototoxicity. The identification of genetic variants that contribute to cisplatin ototoxicity is the first step in the development of predictive diagnostic markers to reduce the incidence of cisplatin ototoxicity, thereby improving treatment outcomes.

Table 3 Combined effect of *TPMT* and *COMT* genotypes on cisplatin-induced hearing loss

Gene	SNP	Genotype	Discovery ($n = 53$)			Replication ($n = 109$)			Combined ($n = 162$)							
			Ototox ^a ($n = 33$)	Controls ^a ($n = 20$)	OR	Ototox ^a ($n = 73$)	Controls ^a ($n = 36$)	OR	Ototox ^a ($n = 106$)	Controls ^a ($n = 56$)	OR	P value ^b	Sens	Spec	PPV	NPV
<i>TPMT</i>	rs12201199	A/_	9 (27.3%)	0 (0.0%)	15.9	16 (21.9%)	1 (2.8%)	9.8	25 (23.6%)	1 (1.8%)	17.0	0.000181	23.6%	98.2%	96.2%	40.4%
		T/T	24 (72.7%)	20 (100%)		57 (78.1%)	35 (97.2%)		81 (76.4%)	55 (98.2%)						
<i>COMT</i>	rs9332377	A/_	13 (39.4%)	2 (10.0%)	5.8	18 (24.7%)	2 (5.6%)	5.6	31 (29.2%)	4 (7.1%)	5.4	0.00109	29.2%	92.9%	88.6%	40.9%
		G/G	20 (60.6%)	18 (90.0%)		55 (75.3%)	34 (94.4%)		75 (70.8%)	52 (92.9%)						
Unique carriers of either ^c			20 (60.6%)	2 (10.0%)	13.8	31 (42.5%)	2 (5.6%)	12.5	51 (48.1%)	4 (7.1%)	12.1	3.4×10^{-8}	48.1%	92.9%	92.7%	48.6%
Non-carriers			13 (39.4%)	18 (90.0%)		42 (57.5%)	34 (94%)		55 (51.9%)	52 (92.9%)						

^aNumber of individuals (percentage of the entire group). ^bDetermined using Fisher exact test. ^cCombination of unique carriers of either *TPMT* risk genotype (rs12201199 A/_) or *COMT* risk genotype (rs9332377 A/_). Ototox, individuals with ototoxicity. Sens, sensitivity; spec, specificity; PPV, positive predictive value; NPV, negative predictive value.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturegenetics/>.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

We especially want to acknowledge the study participants and their families for their participation in the Canadian Pharmacogenomics Network for Drug Safety (CPNDS) network; this work could not have been done without your help and support. We also want to acknowledge the support of the CPNDS active ADR surveillance consortium, as well as S. Langlois and B. Casey (**Supplementary Note**). The study was funded by Genome Canada, and additional funding was also provided by Genome British Columbia, Child and Family Research Institute, University of British Columbia Faculty of Pharmaceutical Sciences, Canadian Institutes of Health Research, Canada Foundation for Innovation, Canada Gene Cure Foundation, Canadian Society of Clinical Pharmacology, BC Clinical Genomics Network, C17 Research Network and Childhood Cancer Foundation—Candlelighters Canada, Michael Smith Foundation for Health Research, Health Canada, Pfizer, Eli Lilly, Merck Frosst and Janssen-Ortho. This work was funded as part of the peer-reviewed Genome Canada Applied Health Research Program; the pharmaceutical industry partners had no formal or informal role in this program of research.

AUTHOR CONTRIBUTIONS

B.C.C., S.R.R., M.J.R., P.C.R. and members of the CPNDS consortium recruited subject cohorts. B.B., B.C.C. and S.R.R. phenotyped subject cohorts. C.J.D.R., H.K.-E., H.V., A.M.K.B. and M.S.P. designed and performed genotyping studies. C.J.D.R., H.K.-E., H.V., A.B., Y.F.-Z. and M.-P.D. analyzed genotyping data. B.C.C. and M.R.H. planned and coordinated the study. All authors contributed to the final version of the manuscript.

Published online at <http://www.nature.com/naturegenetics/>.

Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions/>.

- Brock, P. & Bellman, S. Ototoxicity of cisplatin. *Br. J. Cancer* **63**, 159–160 (1991).
- Li, Y., Womer, R.B. & Silber, J.H. Predicting cisplatin ototoxicity in children: the influence of age and the cumulative dose. *Eur. J. Cancer* **40**, 2445–2451 (2004).
- Coradini, P.P., Cigana, L., Selistre, S.G., Rosito, L.S. & Brunetto, A.L. Ototoxicity from cisplatin therapy in childhood cancer. *J. Pediatr. Hematol. Oncol.* **29**, 355–360 (2007).
- Knight, K.R., Kraemer, D.F. & Neuwelt, E.A. Ototoxicity in children receiving platinum chemotherapy: underestimating a commonly occurring toxicity that may influence academic and social development. *J. Clin. Oncol.* **23**, 8588–8596 (2005).
- Kushner, B.H., Budnick, A., Kramer, K., Modak, S. & Cheung, N.K. Ototoxicity from high-dose use of platinum compounds in patients with neuroblastoma. *Cancer* **107**, 417–422 (2006).
- Oldenburg, J., Kraggerud, S.M., Cvancarova, M., Lothe, R.A. & Fossa, S.D. Cisplatin-induced long-term hearing impairment is associated with specific glutathione S-transferase genotypes in testicular cancer survivors. *J. Clin. Oncol.* **25**, 708–714 (2007).
- Peters, U. *et al.* Glutathione S-transferase genetic polymorphisms and individual sensitivity to the ototoxic effect of cisplatin. *Anti-Cancer Drugs. Anticancer Drugs* **11**, 639–643 (2000).
- Riedemann, L. *et al.* Megalin genetic polymorphisms and individual sensitivity to the ototoxic effect of cisplatin. *Pharmacogenomics J.* **8**, 23–28 (2008).
- Blakley, B.W., Gupta, A.K., Myers, S.F. & Schwan, S. Risk factors for ototoxicity due to cisplatin. *Arch. Otolaryngol. Head Neck Surg.* **120**, 541–546 (1994).
- Bess, F.H., Dodd-Murphy, J. & Parker, R.A. Children with minimal sensorineural hearing loss: prevalence, educational performance, and functional status. *Ear Hear.* **19**, 339–354 (1998).
- Ekborn, A. *et al.* Cisplatin-induced hearing loss: influence of the mode of drug administration in the guinea pig. *Hear. Res.* **140**, 38–44 (2000).
- Hirschhorn, J.N. & Daly, M.J. Genome-wide association studies for common diseases and complex traits. *Nat. Rev. Genet.* **6**, 95–108 (2005).
- Ujiiie, S., Sasaki, T., Mizugaki, M., Ishikawa, M. & Hiratsuka, M. Functional characterization of 23 allelic variants of thiopurine S-methyltransferase gene (TPMT*2 - *24). *Pharmacogenet. Genomics* **18**, 887–893 (2008).
- Diatchenko, L. *et al.* Genetic basis for individual variations in pain perception and the development of a chronic pain condition. *Hum. Mol. Genet.* **14**, 135–143 (2005).
- Nackley, A.G. *et al.* Human catechol-O-methyltransferase haplotypes modulate protein expression by altering mRNA secondary structure. *Science* **314**, 1930–1933 (2006).
- Price, A.L. *et al.* Principal components analysis corrects for stratification in genome-wide association studies. *Nat. Genet.* **38**, 904–909 (2006).
- Visscher, H. *et al.* Application of principal component analysis to pharmacogenomic studies in Canada. *Pharmacogenomics J.* advance online publication, doi:10.1038/tj.2009.36 (4 August 2009).
- The International HapMap Consortium. A haplotype map of the human genome. *Nature* **437**, 1299–1320 (2005).
- Shord, S.S., Thompson, D.M., Krempf, G.A. & Hanigan, M.H. Effect of concurrent medications on cisplatin-induced nephrotoxicity in patients with head and neck cancer. *Anticancer Drugs* **17**, 207–215 (2006).
- Krynetski, E.Y., Krynetskaia, N.F., Yanishevski, Y. & Evans, W.E. Methylation of mercaptopurine, thioguanine, and their nucleotide metabolites by heterologously expressed human thiopurine S-methyltransferase. *Mol. Pharmacol.* **47**, 1141–1147 (1995).
- Yates, C.R. *et al.* Molecular diagnosis of thiopurine S-methyltransferase deficiency: genetic basis for azathioprine and mercaptopurine intolerance. *Ann. Intern. Med.* **126**, 608–614 (1997).
- Relling, M.V. *et al.* Mercaptopurine therapy intolerance and heterozygosity at the thiopurine S-methyltransferase gene locus. *J. Natl. Cancer Inst.* **91**, 2001–2008 (1999).
- Weinshilboum, R.M. Pharmacogenomics: catechol O-methyltransferase to thiopurine S-methyltransferase. *Cell. Mol. Neurobiol.* **26**, 539–561 (2006).
- Weinshilboum, R.M. & Sladek, S.L. Mercaptopurine pharmacogenetics: monogenic inheritance of erythrocyte thiopurine methyltransferase activity. *Am. J. Hum. Genet.* **32**, 651–662 (1980).
- Ochoa, B., Bobadilla, N., Arrellin, G. & Herrera, L.A. S-Adenosyl-L-methionine increases serum BUN and creatinine in cisplatin-treated mice. *Arch. Med. Res.* **40**, 54–58 (2009).
- Ahmed, Z.M. *et al.* Mutations of LRTOMT, a fusion gene with alternative reading frames, cause nonsyndromic deafness in humans. *Nat. Genet.* **40**, 1335–1340 (2008).
- Du, X. *et al.* A catechol-O-methyltransferase that is essential for auditory function in mice and humans. *Proc. Natl. Acad. Sci. USA* **105**, 14609–14614 (2008).
- Nelson, M.R. *et al.* Genome-wide approaches to identify pharmacogenetic contributions to adverse drug reactions. *Pharmacogenomics J.* **9**, 23–33 (2008).
- Link, E. *et al.* SLC01B1 variants and statin-induced myopathy—a genomewide study. *N. Engl. J. Med.* **359**, 789–799 (2008).

ONLINE METHODS

Subjects. Study participants were recruited by the Canadian Pharmacogenomics Network for Drug Safety (CPNDS), a national multicenter active surveillance consortium for studying adverse drug reactions in children³⁰. In the first phase of the study, individuals with cisplatin-induced serious hearing loss and drug-matched controls who received cisplatin but did not suffer hearing loss were recruited from the BC Children's Hospital, Vancouver, Canada. A second cohort of children treated in pediatric oncology units were recruited from across Canada: Alberta Children's Hospital (Calgary, Alberta), Stollery Children's Hospital (Edmonton, Alberta), Winnipeg Health Sciences Centre (Winnipeg, Manitoba), London Health Sciences Centre (London, Ontario), Hospital for Sick Children (Toronto, Ontario), Kingston General Hospital (Kingston, Ontario), Children's Hospital of Eastern Ontario (Ottawa, Ontario), Hôpital Sainte-Justine (Montreal, Quebec), IWK Health Centre (Halifax, New Brunswick) and McMaster Children's Hospital (Hamilton, Ontario). Cisplatin-induced ototoxicity was diagnosed on the basis of audiometric findings using criteria described by the CTCAE (Cancer Therapy Evaluation Program, Common Terminology Criteria for Adverse Events) Version 3 (**Supplementary Table 2**). All subject data were reviewed blind to genotype data by a clinical pharmacologist, an audiologist, an oncologist and an ADR surveillance clinician who reviewed audiogram test results and medical records. Subjects with serious cisplatin ototoxicity were defined as those with grade 2 or higher CTCAE hearing impairment after treatment with cisplatin. Grade 2–3 hearing impairment is the point at which cisplatin chemotherapy protocols recommend halting or reducing cisplatin doses. Controls included children treated in pediatric oncology units who did not develop hearing impairment (grade 0). The high incidence of serious ototoxicity limited the enrollment of control subjects. Informed written consent was obtained from each subject, and the study was approved by ethics committees of all participating universities and hospitals.

An anonymized cohort of 192 unrelated children with a clinical history of severe hearing loss that was not induced by cisplatin were recruited from the British Columbia Children's Hospital to determine the frequency of cisplatin-ototoxic genetic variants in a pediatric population with hearing impairment. The analysis of this anonymized cohort was approved by the ethics committees of the University of British Columbia and British Columbia's Children's Hospital.

Genotyping. Genomic DNA was purified using the QIAamp DNA purification system (Qiagen). DNA samples were genotyped for 1,949 SNPs using a customized Illumina GoldenGate SNP genotyping assay (Illumina) designed to capture the genetic variation of 220 key drug metabolism genes (that is, phase I and II drug metabolism enzymes, drug transporters, drug targets, drug receptors, transcription factors, ion channels and other disease-specific genes related to the physiological pathway of cisplatin). All SNP genotype data were manually clustered using BeadStudio software (Illumina). SNPs that could not be clustered or were nonpolymorphic were excluded from further analyses. The ADME panel consisted of 597 functional SNPs, identified primarily by literature review, that cause nonsynonymous amino acid changes or have been associated with changes in enzyme activity or function. The panel also consisted of 1,352 tag SNPs identified using the ldSelect algorithm to select a maximally informative set of tag SNPs to assay in the candidate genes³¹. The tag SNP selection was performed using data from the International HapMap project that included all four populations (CEU, CHB, JPT and YRI) with a threshold for the LD statistic r^2 of 0.8 and a minor allele frequency of more than 0.05. The concordance of genotype calls between replicate genotyped samples was greater than 99.9% ($n = 25$). The average genotyping call rate

for all samples was 98.8%. If the call rate for any sample was below 95%, the sample was excluded from further analysis.

Statistical analysis. Hardy-Weinberg equilibrium tests were conducted using the permutation version of the exact test of Hardy-Weinberg of Guo and Thompson³². We adjusted for multiple testing using the simpleM correction³³ and calculated the effective number of independent tests (M_{effG}) at 147, for a significance threshold of 0.00034. Thirteen SNPs were removed due to Hardy-Weinberg disequilibrium and 133 SNPs with <0.90 completion were removed, leaving 1,803 SNPs for analysis. Case-control tests of association for the genotypic (2 d.f.), allelic (1 d.f.) and Armitage trend tests (1 d.f.) were performed on the discovery cohort using SAS/Genetics release 9.1 (SAS Institute Inc.). The average identity by state was computed for each subject pair as the sum of the number of identical-by-state alleles at each locus divided by twice the number of loci. We found no duplicated ($\geq 98\%$ identity) or related individuals (86–97%). Principal component analysis was used to assess the population structure in the dataset¹⁶. Graphical display of principal components were prepared with the HelixTree software using the EIGENSTRAT method. We used forward selection in logistic regression testing for the first principal component, sex, age, cisplatin dose and treatment duration. None of the variables were retained in the discovery cohort at a threshold of 0.10, but gender ($P = 0.0425$) and age ($P = 0.086$) were entered in the combined cohort.

We calculated homozygous and heterozygous odds ratios (OR) using the homozygous genotype of the protective allele as reference. OR computations in the presence of empty cells were adjusted by adding 0.5 to all cells. Sensitivity was measured to assess how well the heterozygous, homozygous or combined genotypes can correctly classify ototoxicity cases. Similarly, specificity was measured to assess how well the genotypes can correctly classify controls. Positive predicted value (PPV) was calculated as the proportion of subjects with the ototoxicity-associated genotypes with ototoxicity, and negative predicted value (NPV) was calculated as the proportion of subjects without the ototoxicity-associated genotype and without ototoxicity.

DNA sequencing. The coding regions and intron-exon splice sites of *TPMT* and *COMT* were sequenced using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). An additional 1,000 bp were sequenced upstream and downstream of each gene, providing coverage of the *TPMT* promoter and the distal (membrane bound) as well as the proximal (cytosolic) promoters of *COMT*. PCR and sequencing primers were designed using ExonPrimer software, primers were synthesized by Invitrogen and PCR reaction products were purified by QIAquick 96 PCR Purification Kit (Qiagen, C. Sequence data were analyzed using the Phred/Phrap/Consed software package (Genome Software Development, University of Washington, Seattle, Washington, USA).

URLs. ldSelect, <http://droog.gs.washington.edu/ldSelect.html>; ExonPrimer, <http://ihg2.helmholtz-muenchen.de/ihg/ExonPrimer.html>.

30. Ross, C.J. *et al.* Genotypic approaches to therapy in children: a national active surveillance network (GATC) to study the pharmacogenomics of severe adverse drug reactions in children. *Ann. NY Acad. Sci.* **1110**, 177–192 (2007).
31. Carlson, C.S. *et al.* Selecting a maximally informative set of single-nucleotide polymorphisms for association analyses using linkage disequilibrium. *Am. J. Hum. Genet.* **74**, 106–120 (2004).
32. Guo, S.W. & Thompson, E.A. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics* **48**, 361–372 (1992).
33. Gao, X., Starmer, J. & Martin, E.R. A multiple testing correction method for genetic association studies using correlated single nucleotide polymorphisms. *Genet. Epidemiol.* **32**, 361–369 (2008).