Identification of Genetic Variants Contributing to Cisplatin-Induced Cytotoxicity by Use of a Genomewide Approach

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Cisplatin, a platinating agent commonly used to treat several cancers, is associated with nephrotoxicity, neurotoxicity, and ototoxicity, which has hindered its utility. To gain a better understanding of the genetic variants associated with cisplatin-induced toxicity, we present a stepwise approach integrating genotypes, gene expression, and sensitivity of HapMap cell lines to cisplatin. Cell lines derived from 30 trios of European descent (CEU) and 30 trios of African descent (YRI) were used to develop a preclinical model to identify genetic variants and gene expression that contribute to cisplatin-induced cytotoxicity in two different populations. Cytotoxicity was determined as cell-growth inhibition at increasing concentrations of cisplatin for 48 h. Gene expression in 176 HapMap cell lines (87 CEU and 89 YRI) was determined using the Affymetrix GeneChip Human Exon 1.0 ST Array. We identified six, two, and nine representative SNPs that contribute to cisplatin-induced cytotoxicity through their effects on 8, 2, and 16 gene expressions in the combined, Centre d'Etude du Polymorphisme Humain (CEPH), and Yoruban populations, respectively. These genetic variants contribute to 27%, 29%, and 45% of the overall variation in cell sensitivity to cisplatin in the combined, CEPH, and Yoruban populations, respectively. Our whole-genome approach can be used to elucidate the expression of quantitative trait loci contributing to a wide range of cellular phenotypes.

Cisplatin, a platinating agent, is commonly used to treat head and neck, testicular, lung, and gynecological cancers.[1–3] It has been shown that cisplatin exerts its antitumor activity by binding preferentially to the nucleophillic positions on guanine and adenine of DNA, resulting in the formation of intra- and interstrand crosslinks. Eventually, crosslinks lead to DNA-strand breaks and, ultimately, to cell death.[4,5] Despite its wide usage, dose-limiting toxicities—in particular, nephrotoxicity[6] and neurotoxicity[7]—have hindered the utility of this agent. In addition, treatment-induced ototoxicity can result in dose reduction or discontinuation of cisplatin treatment.[8] The incidences of cisplatin treatment–induced toxicities are highly variable and are associated with cumulative treatments or dose intensities.[7]

High levels of drug efflux transporters, detoxifiers, and DNA-repair proteins and a low Bax:Bcl-2 ratio have all been suggested to play a role in cisplatin resistance.[9] Genetic variants in candidate genes have demonstrated an association with clinical response to or toxicity from cisplatin. For example, two common SNPs of *ERCC1* are correlated with an increased risk of toxicity and with the survival of cisplatin-treated patients with non–small-cell lung cancer.[10,11] Polymorphisms in cytokine-promoter genes (e.g., *TNF, IL1, IL6*) have been suggested to be associated with toxicities induced by treatment with 5-fluorouracil and cisplatin.[12] Glutathione S-transferase genetic polymorphisms have also been associated with treatment outcomes of paclitaxel- and cisplatin-based chemotherapy.[13] An illustration of candidate genes involved in the mechanism of cisplatin activity can be found at the PharmGKB Web site. Although the study of candidate genes and pathways has increased our understanding of the mechanism of action of platinating agents, our understanding of genetic variants important in determining a patient's likelihood of response or toxicity is extremely limited. Thus, the development of comprehensive, unbiased models is critical to the identification of genetic variants and genes contributing to interindividual variation in drug effect. Genomewide approaches open up the possibility of identifying genetic and/or expression signatures that can be evaluated in clinical trials, for validation.

Previously, we used Epstein-Barr virus (EBV)–transformed B-lymphoblastoid cell lines (LCLs) derived from healthy individuals within 10 large CEPH pedigrees and demonstrated that 38%–47% of human variation in susceptibility to cisplatin-induced cytotoxicity is due to genetic components.[14] To better elucidate the genetic variants important in cisplatin-induced cytotoxicity, we employed a genomewide association study, using the International HapMap cell lines derived from trios of northern and western European and Yoruban populations. These

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well-genotyped samples provide an extremely rich data set for genotype–drug effect correlations.[15] We performed gene-expression analysis on these HapMap cell lines, using the Affymetrix GeneChip Human Exon 1.0 ST Array, and phenotyped the samples for susceptibility to cisplatin-induced cytotoxicity. The focus of this article is the description of genetic variants in two populations that contribute, through variation in gene expression, to cisplatin-induced cytotoxicity. To this end, we designed a three-way model, correlating genotype, gene expression, and cytotoxicity data, to identify potentially functional SNPs and/or haplotypes associated with cisplatin-induced cytotoxicity. Cell lines derived from individuals of European and African descent allow us to define a set of genetic variants unique to and common among the populations. The long-term goal is to identify, through a genetic signature, patients at risk for adverse events associated with these agents.

Material and Methods

Material

EBV-transformed LCLs derived from 30 CEPH trios (i.e., mother, father, and child) collected from Utah residents with northern and western European ancestry (CEU [HAPMAPPT01]) and from 30 trios collected from Yoruba in Ibadan, Nigeria (YRI [HAPMAPPT03]), were purchased from the Coriell Institute for Medical Research. Cell lines were maintained and were diluted as described elsewhere.[16] Cisplatin and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich.

Cytotoxicity Assay

Cell-growth inhibition was evaluated at concentrations of 0, 0.5, 1, 2.5, 5, 10, 20, 40, and 80 μM of cisplatin. Cisplatin was dissolved in DMSO immediately before use. DMSO concentrations did not exceed 0.1% in the cells. The cytotoxic effect of cisplatin was determined using the nontoxic colorimetric-based assay alamarBlue, as described elsewhere.[16] The concentration required to inhibit 50% of cell growth (IC_{50}) was determined by curve fitting the percentage of cell survival against the concentration of cisplatin.

Genotype and Cytotoxicity Association Analysis

SNP genotypes were downloaded from the International HapMap database (release 21). To perform a high-quality genomewide association study, we employed several data filters. To reduce possible genotyping errors, we excluded 100,536 and 138,533 SNPs with Mendelian allele-transmission errors in 22 autosomes in the 30 CEU and 30 YRI HapMap trios, respectively. To exclude the extreme outliers and to increase the power of the association studies within our limited number of samples, we included only the SNPs that met the criteria of having three genotypes and containing a minimum of two counts for each genotype in the unrelated individuals of each population. To obtain functionally relevant SNPs, we further filtered the SNPs by their location. Only SNPs located in genes or within 10 kb up- or downstream of a gene were included. Thus, our final data set consisted of 387,417 very informative SNPs covering 22,667 well-annotated genes.

All 175 IC_{50} values (from 86 CEU and 89 YRI) were $\log_2$ transformed to obtain normally distributed data. The quantitative transmission/disequilibrium test (QTDT) was performed to identify any genotype-cytotoxicity associations, with the use of QTDT software.[17] Because of the possible heterogeneity between and within populations, we performed association studies in these two ethnic groups separately, using sex as a covariate, and together, using sex and race as covariates. $P \leqslant .0001$ was considered statistically significant.

Gene-Expression Assessment

RNA from 87 CEU and 89 YRI cell lines was extracted after four dilutions, by use of RNeasy Plus Mini Kits (QIAGEN). RNA quality was assessed using the RNA 6000 Nano Assay (Agilent Technologies). For each cell line, ribosomal RNA was depleted from 1 μg of total RNA by use of the RiboMinus Human/Mouse Transcriptome Isolation Kit (Invitrogen). cDNA was generated using the GeneChip WT cDNA Synthesis and Amplification Kit (Affymetrix), per the manufacturer's instructions. cDNA was fragmented and end labeled using the GeneChip WT Terminal Labeling Kit (Affymetrix). Approximately 5.5 μg of labeled DNA target was hybridized to the Affymetrix GeneChip Human Exon 1.0 ST Array at 45°C for 16 h, per the manufacturer's recommendation (see Affymetrix Web site for additional information). Hybridized arrays were washed and stained on a GeneChip Fluidics Station 450 and were scanned on a GCS3000 Scanner (Affymetrix). Resulting probe-signal intensities were sketch-quantile normalized using a subset of the 1.4 million probe sets. Gene-expression levels were summarized using the robust multiarray average (RMA). A constant of 16 was added for variance stabilization, and summarized signals were $\log_2$ transformed.[18] This was done with signals generated on a core set of well-annotated exons (~200,000) within the Affymetrix Exon Array Computational Tool (ExACT) software package. To prevent confounding interpretations of gene-expression variation, we removed data from exons for which probe sets contained two or more probes harboring SNPs, before summarizing expression. All raw exon-array data have been deposited into Gene Expression Omnibus (GEO) (accession number GSE7761).

Genotype and Gene-Expression Association Analysis

A second QTDT test that integrated mRNA gene expression and significant SNPs found in the genotype and cytotoxicity association analysis was performed to identify possible association with gene expression. Significant SNPs generated from the genotype-cytotoxicity association in CEU, YRI, or combined populations were tested for their association with gene expression in the same population. Genes with average intensity >5 from Affymetrix GeneChip Human Exon 1.0 ST Array analysis were considered expressed and were included in this association analysis. The QTDT test was performed using gene-expression analysis in CEU and YRI populations separately and combined, with sex and race as covariates in the combined samples. We examined not only the *cis*-acting gene, defined as gene expression associated with SNP(s) within 5 Mb on the same chromosome, but also the *trans*-acting gene, defined as gene expression associated with SNP(s) on different chromosome(s) or >5 Mb away on the same chromosome. A Bonferroni correction ($P < .05$) that used a number of transcript clusters in the analysis was used to adjust raw P values after QTDT analysis.

Gene Expression and Cisplatin IC_{50} Linear-Regression Analysis

To examine the relationship between gene expression and sensitivity to cisplatin, we constructed a general linear model with $\log_2$-transformed cisplatin IC_{50} as the dependent variable and RMA-summarized $\log_2$-transformed gene-expression level and an indicator for sex as the independent variables. The dependent variable was transformed to satisfy the assumption of normality. Trios were treated as units of analysis, and members of different families were considered independent. The covariance structure within a trio was modeled using a Toeplitz structure with two diagonal bands, such that the trios were ordered father, then offspring, and then mother. With this covariance structure, mother and father IC_{50} values were independent, but the offspring's value was allowed to covary with both the father's and mother's values. If a SNP was significantly associated with cisplatin IC_{50} and the same SNP was significantly associated with gene expression, then the above approach was used to test whether gene expression significantly predicted IC_{50}. In the CEU population, 4 transcript clusters were tested for their expression correlation with cisplatin IC_{50}, whereas 19 transcript clusters were tested in the YRI population, and 19 transcript clusters were tested in the combined CEU and YRI populations. With the combined approach, predictors of population and sex were included in the model. Sex was also tested in the separate CEU and YRI populations as a predictor of cisplatin IC_{50}. $P < .05$ was considered statistically significant. The model was programmed using the PROC MIXED procedure in SAS/STAT software version 9.1.[19] The REPEATED statement was used to model the Toeplitz covariance structure. The linkage disequilibrium (LD) of significant SNPs within each population was evaluated using Haploview version 3.32.

Multivariate Model to Predict Association of Cisplatin IC_{50} with Genotypes

To examine the overall genetic contributions to sensitivity of cisplatin, additional general linear models were constructed with transformed cisplatin IC_{50} as the dependent variable. The independent variables included all the significant SNP genotypes (with assumption of an additive genetic effect) that were selected using the three-way model in the combined populations and in the two populations independently. These SNP genotypes were significantly associated with cisplatin IC_{50} through their effect on gene expression. For the model of combined populations, indicators of race and sex were also included as predictors. Trios were analyzed as independent units. The covariance was modeled as described above. Models were reduced using a backward-elimination approach. SNPs included in each of the final models were statistically significant at the $\alpha = .05$ level. By use of the final model, predicted transformed IC_{50} values were computed. For the unrelated individuals (parents from the trios and, separately, offspring from the trios), R^2 was estimated between observed IC_{50} and the predicted IC_{50} from the final model. Lastly, a weighted average of the two R^2 estimates was computed to quantify the amount of variation in cisplatin IC_{50} explained by the selected SNP genotypes.

Alternative Methods

Alternative methods were considered to evaluate the endpoints of the analytical experiments with use of a different initial step but with the same statistical cutoffs. The first alternative approach involved analyzing the SNP genotype and gene-expression association, testing the association of the significant SNPs with cisplatin IC_{50}, and then performing linear regression between gene expression and cisplatin IC_{50}. The second alternative approach involved evaluating the correlation between gene expression and cisplatin IC_{50}, followed by analyzing the SNPs associated with gene expression and testing the association between those SNPs and cisplatin IC_{50}.

Results

QTDT Genotype-Cytotoxicity Association

Elsewhere, we reported the median IC_{50} as 5.1 μM and 6.3 μM for cell lines derived from CEU ($n = 86$) and YRI ($n = 89$) trios, respectively, after exposure to increasing concentrations of cisplatin (0.5–80 μM) for 48 h.[16] Interindividual variation in the IC_{50} was 17-fold for CEU and 49-fold for YRI.[16] Using 387,417 SNPs representing 22,667 genes (~85% of genes in the entire genome), we evaluated whether genetic variation was associated with sensitivity to cisplatin by use of the IC_{50} value. An arbitrary P value threshold ($P \leq .0001$) resulted in the identification of 96, 57, and 138 SNPs significantly associated with cisplatin IC_{50} in the combined, CEU, and YRI populations, respectively (table 1). These SNPs were located in or within 10 kb up- or downstream of 67, 36, and 88 genes, respectively.

QTDT Genotype and Gene-Expression Association

We generated expression data on 176 LCLs (87 CEU and 89 YRI), using the Affymetrix GeneChip Human Exon 1.0 ST Array (an exon array). A total of 14,722 transcript clusters with a mean $\log_2$-transformed gene-expression intensity of >5, indicating expression in both CEU and YRI samples, were included in the analysis. The QTDT association analysis was conducted between gene expression and the SNPs that were significantly associated with cisplatin IC_{50}. We found 2 *cis*- and 32 *trans*-acting relationships in the combined populations, 1 *cis*- and 3 *trans*-acting relationships in CEU, and 2 *cis*- and 36 *trans*-acting relationships in YRI (Bonferroni-corrected $P < .05$). Among all observed *cis*- and *trans*-acting relationships, some SNPs were significantly associated with more than one gene expression, and some gene expressions were associated with more than one SNP. Therefore, the final *cis*- and *trans*-acting relationships were represented by 8 SNPs that were significantly associated with 22 gene expressions in the combined population, by 3 SNPs that were significantly associated with 4 gene expressions in CEU, and by 11 SNPs that were significantly associated with 25 gene expressions in YRI (table 1 and the tab-delimited ASCII file, which can be imported into a spreadsheet, of data set 1 [online only]).

Linear Regression of Gene Expression and Cisplatin IC_{50}

We examined the correlation between gene expression and cisplatin IC_{50}, using a general linear model that was constructed to reflect the trio relationships in our data.

Table 1. Significant Results from the Three-Way Model with Combined, CEU, and YRI Populations

Approach and Steps	No. of SNPs (No. of Genes)		
	Combined Populations	CEU	YRI
Current:			
SNP associated with cisplatin IC_{50}[a]	96 (67)	57 (36)	138 (88)
SNP associated with gene expression[b]	8 (22)	3 (4)	11 (25)
Gene expression correlated with cisplatin IC_{50}[c]	6 (8)	2 (2)	10 (17)
Alternative 1:			
SNP associated with gene expression[b]	20,440 (8,451)	16,284 (5,922)	23,787 (9,059)
SNP associated with cisplatin IC_{50}[a]	8 (22)	3 (4)	11 (25)
Gene expression correlated with cisplatin IC_{50}[c]	6 (8)	2 (2)	10 (17)
Alternative 2:			
Gene expression correlated with cisplatin IC_{50}[c]	NA (2,934)	NA (1,770)	NA (1,882)
SNP associated with gene expression[b]	16,129 (2,378)	11,576 (1,311)	21,456 (1,522)
SNP associated with cisplatin IC_{50}[a]	22[d] (19)	8[e] (8)	36[f] (24)

NOTE.—NA = not applicable.

[a] $P \leq .0001$.

[b] Bonferroni-corrected $P < .05$.

[c] $P < .05$.

[d] The additional SNPs generated from this approach are *rs10825264, rs10894795, rs12049577, rs12278731, rs13278343, rs2484665, rs3123678, rs3886003, rs6436716, rs6552924, rs7013683, rs7699288, rs773921, rs7795668, rs7825213,* and *rs979532*.

[e] The additional SNPs generated from this approach are *rs10898290, rs1556223, rs1953951, rs1975092, rs2111890,* and *rs2276607*.

[f] The additional SNPs generated from this approach are *rs1004407, rs10053097, rs10221083, rs10431791, rs12499960, rs1291362, rs17740395, rs1889785, rs2017791, rs4474730, rs6043976, rs6043979, rs6043981, rs6043984, rs6043986, rs6974263, rs7226876, rs8045919, rs8051159, rs850920, rs940795, rs9455158, rs981890, rs9821880, rs9881766,* and *rs9882242*.

Since some genes shared the same transcript cluster identification numbers (IDs) on the exon array, the expression of 19 transcript clusters (representing 22 genes identified above) were evaluated in the combined population. Eight genes had significant correlation with cisplatin IC_{50} ($P < .05$) (table 1). In the same manner, we found 2 and 17 genes whose expression significantly correlated with cisplatin IC_{50} in the CEU and YRI populations, respectively ($P < .05$) (table 1, current approach). A summary of SNPs that were found to be significantly associated with cisplatin IC_{50} through gene-expression analysis of the CEU, YRI, and combined populations is shown in table 2.

Alternative methods were considered to evaluate the endpoints of the analytical experiments with use of a different initial step but with the same statistical cutoff. If the initial step is the analysis of the SNP genotype and gene-expression association followed by tests of association of the significant SNPs with cisplatin IC_{50} and linear-regression analysis between gene expression and cisplatin IC_{50} (table 1, alternative approach 1), we find identical results. If, however, the initial step is an analysis of gene expression and cisplatin IC_{50} followed by analysis of the SNP associated with gene expression and then association analysis of those SNPs with cisplatin IC_{50} (table 1, alternative approach 2), the same genetic variants with additional variants are identified. Additional SNPs found through alternative approach 2 are indicated in table 1.

When the results generated from association tests between genotype, cisplatin IC_{50}, and gene expression—as well as the linear-regression results between gene expression and cisplatin IC_{50}—were combined, we identified 6, 2, and 10 SNPs that were significantly associated with cisplatin IC_{50} through regulation of 8, 2, and 17 gene expressions in the combined, CEU, and YRI populations, respectively (table 1). One example was the significant association between the genotype of *rs456998* (located within an intron of the *FCHSD1* gene on chromosome 5) and cisplatin IC_{50} ($P = 4 \times 10^{-5}$) (fig. 1*A*). This SNP was associated with the expression of DNA-damage–inducible transcript 4 (*DDIT4*; on chromosome 10; $P = 2 \times 10^{-6}$); never in mitosis gene A–related kinase 2 (*NEK2* [MIM 604043]; on chromosome 1; $P = 2 \times 10^{-6}$); serine hydroxymethyltransferase, mitochondrial (*SHMT2* [MIM 138450]; on chromosome 12; $P = 1 \times 10^{-6}$); *WDR58* (on chromosome 16; $P = 2 \times 10^{-7}$); and *FRAG1* (on chromosome 11; $P = 3 \times 10^{-6}$) genes (fig. 1*B*), whose expression also significantly correlated with cisplatin IC_{50} in the combined populations ($P = 1 \times 10^{-4}$, 8×10^{-6}, 2×10^{-4}, 7×10^{-4}, and 4×10^{-2}, respectively) (fig. 1*C*).

In addition, we identified a significant association between cisplatin IC_{50} and the genotype of SNP *rs8094647* located in the intron of the myosin Vb gene (*MYO5B* [MIM 606540]; $P = 2 \times 10^{-5}$) on chromosome 18 (fig. 2*A*). This same SNP genotype was significantly associated with the expression of the v-myc avian myelocytomatosis viral oncogene homolog gene (*MYC* [MIM 190080]; $P = 1 \times 10^{-6}$) located on chromosome 8 (fig. 2*B*). The GG genotype of *rs8094647* was associated with higher *MYC* gene ex-

Table 2. SNPs Associated with Cisplatin IC_{50} through Gene-Expression Analysis of the CEU, YRI, and Combined Populations

	Host Gene			Target Gene			*P*			
SNP	Chromosome	SNP Location	Name	Transcript Cluster ID	Name	Chromosome Location	Genotype and IC_{50}	Genotype and Expression	Gene Expression and IC_{50}	Population
rs1649942	10	Intron	*NRG3*	3448088	*BHLHB3*	12p11.23-p12.1	.00007	.000003	.0038237	CEU
rs7550918	1	Promoter	*LOC644852*	2790062	*FLJ32028*	4q31.3	.00008	.000003	.0204	CEU
rs4751143	10	Intron	*EBF3*	3867247	*DBP*	19q13.3	.00006	.0000001	.0342	YRI
rs2305638	3	Intron	*NBEAL2*	2461531	*IRF2BP2*	1q42.3	.0001	.0000002	.0187	YRI
rs6512670	20	Intron	*PARD6B*	2873785	*ALDH7A1*	5q31	.00003	.0000003	.0019804	YRI
rs12278731	11	Intron	*GALNTL4*	3705491	*FAM57A*	17p13.3	.00004	.0000005	.000198	YRI
rs9988868	11	Intron	*GALNTL4*	3705491	*FAM57A*	17p13.3	.00004	.0000005	.000198	YRI
rs935196	15	Intron	*ATP8B4*	4013434	*TAF9L*	Xq13.1-q21.1	.0001	.0000007	.0008489	YRI
rs12278731	11	Intron	*GALNTL4*	2489228	*WDR54*	2p13.1	.00004	.000001	.0143	YRI
rs2587708	2	Intron	*TMEM37*	2946319	*HIST1H4D*	6p21.3	.00004	.000001	.0007479	YRI
rs9988868	11	Intron	*GALNTL4*	2489228	*WDR54*	2p13.1	.00004	.000001	.0143	YRI
rs11236836	11	Tail	*LRRC32*	3995804	*FLJ43855*	16p11.2	.0001	.000002	.000005933	YRI
rs11236836	11	Tail	*LRRC32*	3995804	*SLC6A8*	Xq28	.0001	.000002	.000005933	YRI
rs12278731	11	Intron	*GALNTL4*	2673312	*PFKFB4*	3p22-p21	.00004	.000003	.0004802	YRI
rs12278731	11	Intron	*GALNTL4*	3622386	*GATM*	15q21.1	.00004	.000003	.0104	YRI
rs12278731	11	Intron	*GALNTL4*	3965751	*HDAC10*	22q13.31	.00004	.000003	.00002793	YRI
rs12278731	11	Intron	*GALNTL4*	3965751	*MAPK12*	22q13.33	.00004	.000003	.00002793	YRI
rs12278731	11	Intron	*GALNTL4*	2339786	*KIAA1799*	1p31.3	.00004	.000003	.0026355	YRI
rs12278731	11	Intron	*GALNTL4*	2339786	*PGM1*	1p31	.00004	.000003	.0026355	YRI
rs9988868	11	Intron	*GALNTL4*	3622386	*GATM*	15q21.1	.00004	.000003	.0104	YRI
rs9988868	11	Intron	*GALNTL4*	2339786	*KIAA1799*	1p31.3	.00004	.000003	.0026355	YRI
rs9988868	11	Intron	*GALNTL4*	2339786	*PGM1*	1p31	.00004	.000003	.0026355	YRI
rs9988868	11	Intron	*GALNTL4*	3965751	*HDAC10*	22q13.31	.00004	.000003	.00002793	YRI
rs9988868	11	Intron	*GALNTL4*	3965751	*MAPK12*	22q13.33	.00004	.000003	.00002793	YRI
rs9988868	11	Intron	*GALNTL4*	2673312	*PFKFB4*	3p22-p21	.00004	.000003	.0004802	YRI
rs6537571	10	Promoter	*C10orf64*	2999516	*STK17A*	7p12-p14	.00007	.000002	.0183372	YRI
rs3732103	2	Intron	*PQLC3*	3761451	*HOXB9*	17q21.3	.0001	.000003	.00008451	YRI
rs456998	5	Intron	*FCHSD1*	3645565	*WDR58*	16p13.3	.00004	.0000002	.0006867	Combined
rs173683	5	Intron	*FCHSD1*	3418007	*SHMT2*	12q12-q14	.0001	.0000006	.0001657	Combined
rs173683	5	Intron	*FCHSD1*	3645565	*WDR58*	16p13.3	.0001	.0000009	.0006867	Combined
rs8094647	18	Intron	*MYO5B*	3115504	*MYC*	8q24.12-q24.13	.00002	.000001	.0000000000454	Combined
rs456998	5	Intron	*FCHSD1*	3418007	*SHMT2*	12q12-q14	.00004	.000001	.0001657	Combined
rs456998	5	Intron	*FCHSD1*	3251393	*DDIT4*	10pter-q26.12	.00004	.000002	.00009656	Combined
rs456998	5	Intron	*FCHSD1*	2454444	*NEK2*	1q32.2-q41	.00004	.000002	.000008268	Combined
rs1566347	4	Intron	*SORBS2*	3887117	*PPGB*	20q13.1	.00006	.000002	.0044	Combined
rs2136241	1	Promoter	*CDCA1*	3850445	*CDKN2D*	19p13	.00002	.000003	.0157	Combined
rs456998	5	Intron	*FCHSD1*	3317868	*FRAG1*	11p15.5	.00004	.000003	.0425	Combined
rs7244679	18	Intron	*MYO5B*	3115504	*MYC*	8q24.12-q24.13	.00005	.000003	.0000000000454	Combined

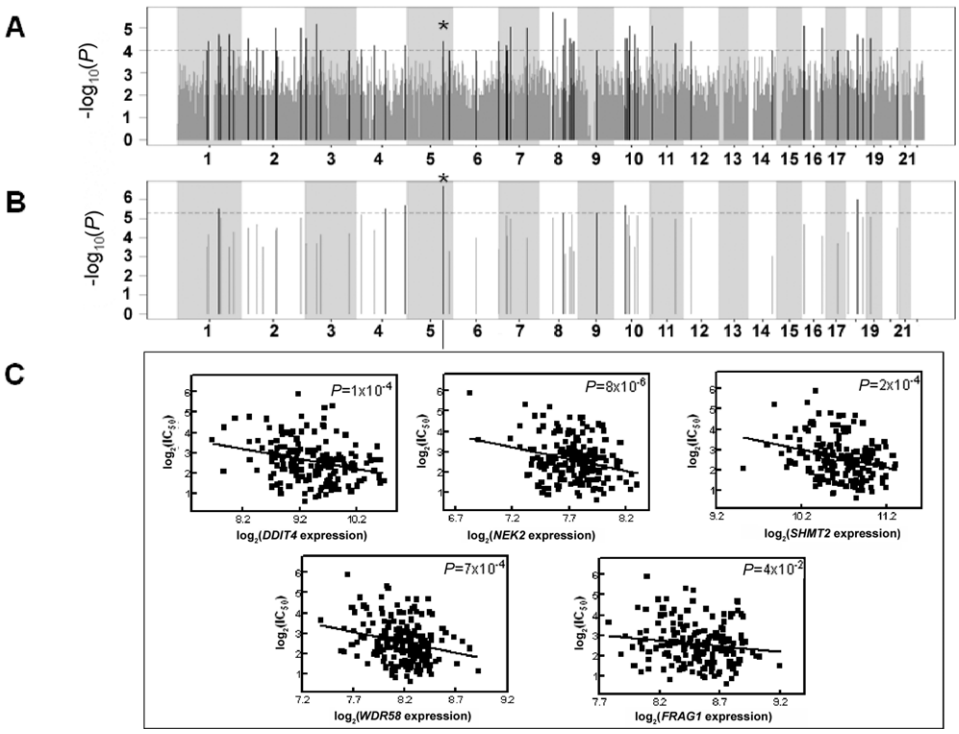

Figure 1. Relationship between SNP genotype, gene expression, and cisplatin IC_{50} in combined CEU and YRI populations. *A,* Genomewide association between SNP genotypes and log_2-transformed cisplatin IC_{50} in the combined populations. The *X*-axis represents the chromosomal location of SNPs. The *Y*-axis represents the statistical significance of association analysis. The dashed line indicates the significance cutoff ($P \leqslant 10^{-4}$). *B,* Association analysis between SNP genotypes and log_2-transformed gene expression in the combined populations. The *X*-axis represents the SNP chromosomal location. The *Y*-axis represents the statistical significance of association analysis. The dashed line indicates the significance cutoff (Bonferroni-corrected $P < .05$). *C,* Correlation between log_2-transformed cisplatin IC_{50} and log_2-transformed *DDIT4, NEK2, SHMT2, WDR58,* and *FRAG1* expression. All five gene expressions are significantly associated with one SNP genotype. This SNP, *rs456998,* is labeled with an asterisk in panels A and B.

pression and lower cisplatin IC_{50}. This was further indicated by the negative correlation found between *MYC* gene expression and cisplatin IC_{50} ($P = 5 \times 10^{-11}$) (fig. 2*C*).

In the individual CEU population, we identified *rs1649942,* located in the intron of the neuregulin 3 gene (*NRG3* [MIM 605533]; $P = 7 \times 10^{-5}$) on chromosome 10, associated with cisplatin IC_{50} (fig. 3*A*) and expression of basic helix-loop-helix domain–containing protein, class B, 3 gene (*BHLHB3* [MIM 606200]; $P = 3 \times 10^{-6}$) (fig. 3*B*). We also found a strong correlation between the *BHLHB3* expression and cisplatin IC_{50} ($P = 4 \times 10^{-3}$) (fig. 3*C*).

In the YRI population, we identified a strong association between the genotype of SNPs *rs12278731* and *rs9988868,* both located in the intron of the *GALNTL4* gene on chromosome 11, and cisplatin IC_{50} ($P = 4 \times 10^{-5}$) (fig. 4*A*). These two SNPs are in complete LD ($D' = 1$; $r^2 = 1$). These SNP genotypes were also significantly associated with the expression of eight genes: *FAM57A* (on chromosome 17; $P = 5 \times 10^{-7}$), *WDR54* (on chromosome 2; $P = 1 \times 10^{-6}$); 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (*PFKFB4* [MIM 605320]; on chromosome 3; $P = 3 \times 10^{-6}$), L-arginine:glycine amidinotransferase (*GATM* [MIM 602360]; on chromosome 15; $P = 3 \times 10^{-6}$), histone deacetylase 10 (*HDAC10* [MIM 608544]; on chromosome 22, $P = 3 \times 10^{-6}$), mitogen-activated protein kinase 12 (*MAPK12* [MIM 602399]; on chromosome 22; $P = 3 \times 10^{-6}$), *KIAA1799* (on chromosome 1; $P = 3 \times 10^{-6}$), and phosphoglucomutase 1 (*PGM1* [MIM 171900]; on chromosome 1; $P = 3 \times 10^{-6}$) (fig. 4*B*). The CC genotype of *rs12278731* was associated with higher expression of all eight genes and lower cisplatin IC_{50}. This was further indicated by the inverse correlation found between these gene expressions and cisplatin IC_{50} (all $P < .05$) (fig. 4*C*).

Multivariate Models to Predict Cisplatin IC_{50} with Genotypes

To examine the overall contributions of our selected genetic variants to sensitivity of cisplatin, we constructed additional general linear models. All SNP genotypes that were significantly associated with cisplatin IC_{50} through their effects on gene expression were included as the independent variables to predict cisplatin IC_{50}, the dependent variable in each population. The backward-elimination approach was applied for model reduction. In the combined population, four of the six tested SNPs were included in the final model ($P < .03$ for all SNPs). Specif-

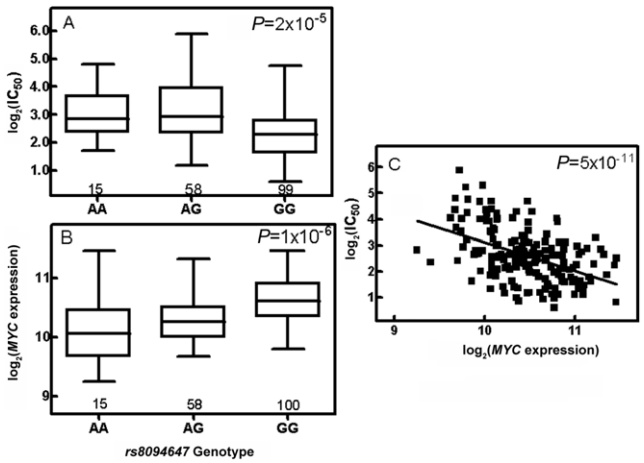

Figure 2. Relationship between *rs8094647, MYC* gene expression, and cisplatin IC_{50} in combined CEU and YRI populations. *A,* Association between *rs8094647* genotype and log_2-transformed cisplatin IC_{50}. *B,* Association between *rs8094647* genotype and log_2-transformed *MYC* expression. *C,* Correlation of log_2-transformed *MYC* expression and log_2 cisplatin IC_{50}. The number of cell lines per genotype is labeled directly above the genotype in panels A and B.

ically, *rs1566347, rs2136241, rs456998,* and *rs7244679* were all significant predictors of cisplatin IC_{50}. The indicator of race is not a significant predictor of cisplatin IC_{50} ($P = .13$). Computing a weighted sum of R^2 from each group of unrelated individuals gives an overall estimate of $R^2 = 0.27$, which indicates that 27% of the variation in cisplatin IC_{50} can be explained by these four SNPs in the combined populations. In the CEU population, both *rs1649942* and *rs7550918* were included in the final model ($P = .002$ and $P = .001$, respectively). The overall estimate of $R^2 = 0.29$ indicates that 29% of the cisplatin IC_{50} variation can be explained by these two SNPs in the CEU population. In the YRI population, 4 of the 10 tested SNPs were included in the final model ($P < .02$ for all SNPs). Specifically, *rs11236836, rs12278731, rs4751143,* and *rs935196* were all significant predictors of cisplatin IC_{50}. The overall estimate of $R^2 = 0.45$ indicates that 45% of the cisplatin IC_{50} variation can be explained by these four SNPs in the YRI population. The indicator of sex is not a significant predictor of cisplatin IC_{50} within CEU ($P = .91$); it is, however, a significant predictor ($P = .03$) in the YRI population, which agrees with our previous finding of a significant difference in cisplatin IC_{50} between female and male subjects within the YRI population.[16]

Discussion

Given the wide usage of cisplatin, the high variability of cisplatin-induced toxicity, and, most importantly, our current inability to use genetic variation to identify patients at risk for toxicity associated with cisplatin, the construction of a genetic model that provides leads for clinical testing should significantly improve the utility of this agent. The leads come from a genomewide, unbiased model that integrates genotype, gene expression, and drug sensitivity. This is the first attempt to integrate, on a genomewide scale, SNP-pattern analysis, gene-expression profiling, and a pharmacologic phenotype (cisplatin-induced cytotoxocity) for the discovery of important, novel genetic information with significant clinical potential. We identified 17 genetic variants significantly associated with cisplatin-induced cytotoxicity through the expression of 26 genes in cell lines derived from the CEU and/or YRI populations. These genetic variants can explain ~27%, ~29%, and ~45% of the overall observed cisplatin IC_{50} variations in the combined, CEU, and YRI populations, respectively.

Using cell lines derived from large pedigrees, our laboratory was the first, to our knowledge, to demonstrate that a significant genetic component contributed to cisplatin susceptibility.[14] More recently, our laboratory has demonstrated that cisplatin-induced cytotoxicity is a highly heritable trait, with 34% of the variation in IC_{50} for 324 LCLs (derived from 27 large CEPH pedigrees) due to genetic factors ($P = 1 \times 10^{-7}$) (S. Shukla, S. Duan, J. Badner, X. Wu, and M. E. Dolan, unpublished data). Linkage analysis revealed suggestive and significant LOD scores >2 at two chromosomal regions. Since no significant population differences in cisplatin-induced cytotoxicity were observed between the CEU and YRI populations, we compared the linkage-scan results obtained from the large CEPH pedigrees with the present association findings.[16] Among all significant SNPs that were found to be associated with cisplatin IC_{50} through gene-expression analysis, two of them are also under suggestive linkage peaks (11p15.4-15.1; LOD >2). These SNPs, *rs12278731* and *rs9988868,* are in complete LD ($D' = 1$; $r^2 = 1$), are located within the intron regions of *GALNTL4* on chromosome 11, and are significantly associated with the expression of eight genes (*FAM57A, WDR54, PFKFB4, GATM, HDAC10, MAPK12, KIAA1799,* and *PGM1*) in the YRI population. The different allele frequencies of the SNPs ($F_{ST} = 0.14$) between the CEU and YRI populations may account for the significant association observed in YRI but not in CEU when our current approach was used.

In the current study, we evaluated the HapMap trios, of which extensive genotypic information provided reasonable power to detect genetic variants that were significantly associated with drug sensitivity. LCLs derived from two HapMap populations, CEU and YRI, were evaluated together and separately to detect significant genetic variants that contribute to cell sensitivity to cisplatin in each population and in both populations combined. Given the stringent statistical cutoffs, we did not observe any overlap of SNPs between the CEU and YRI populations. This was not surprising, given the heterogeneity between the CEU and YRI samples. Nor did we observe SNPs that overlapped between the combined populations and the individual

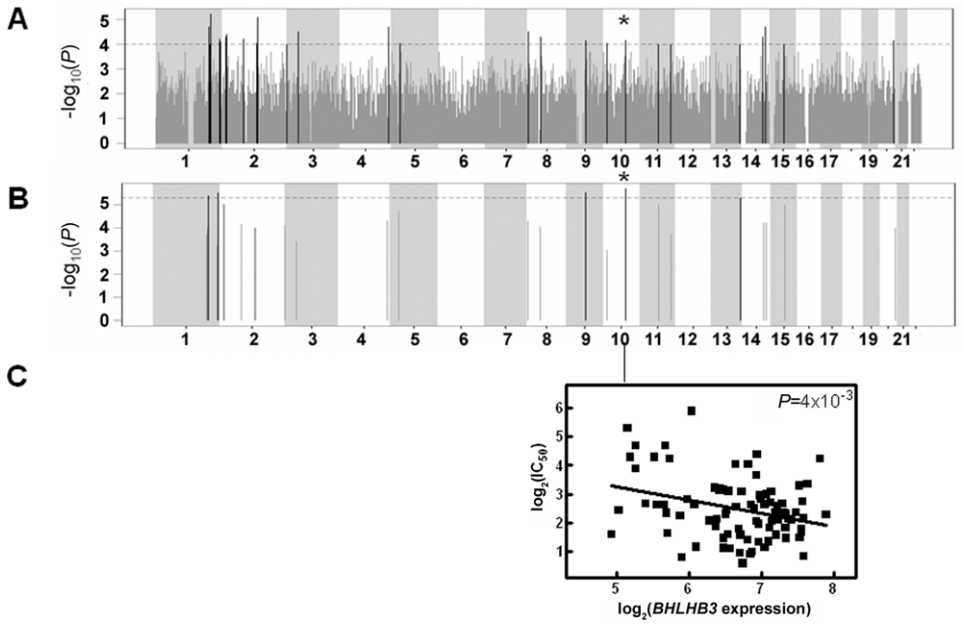

Figure 3. Relationship between SNP genotype, gene expression, and cisplatin IC_{50} in CEU population. *A,* Genomewide association between SNP genotypes and $\log_2$-transformed cisplatin IC_{50} in CEU population. The *X*-axis represents the chromosomal location of SNPs. The *Y*-axis represents the statistical significance of association analysis. The dashed line indicates the significance cutoff ($P \leqslant 10^{-4}$). *B,* Association analysis between SNP genotypes and $\log_2$-transformed gene expression in CEU population. The *X*-axis represents the SNP chromosomal location. The *Y*-axis represents the statistical significance of association analysis. The dashed line indicates the significance cutoff (Bonferroni-corrected $P < .05$). *C,* Correlation between $\log_2$-transformed cisplatin IC_{50} and $\log_2$-transformed *BHLHB3* expression. SNP *rs1649942,* which is significantly associated with cisplatin IC_{50} and *BHLHB3* expression, is labeled with an asterisk in panels A and B.

populations. The larger number of samples in the combined populations provides more power to detect smaller genetic effects on cytotoxicity and gene expression. However, by combining the CEU and YRI data, the unique genetic findings in one population may be masked by the noise produced by the lack of a genetic effect in the other population. Thus, we interpreted the final results in each separate population as population-specific genetic variants that contribute to cisplatin toxicity, whereas those in the combined population are genetic variants important to drug-induced toxicity, regardless of population tested.

Furthermore, our three-step model links genotype, gene expression, and sensitivity to cisplatin, to decrease the potential false-discovery rate. A genomewide association test between genotype and cisplatin IC_{50} yielded 96, 57, and 138 significant SNPs ($P \leqslant .0001$) associated with cisplatin IC_{50} from the combined and CEU- and YRI-derived cell lines, respectively. All these could be functionally important; however, the consideration of a subgroup of those that act through gene expression narrows the list to 6, 2, and 10 SNPs significantly associated with the expression of 8, 2, and 17 genes and significantly correlated with cisplatin IC_{50} ($P < .05$) in combined, CEU, and YRI populations, respectively. Our long-term goal is to identify, before chemotherapy, patients who are at risk for toxicity. Since genotyping is clinically more practical than gene-expression measurements, each approach considered (current and alternatives 1 and 2) results in a genetic signature that considers SNPs that are important in cytotoxicity and expression. The first alternative approach is also a SNP-oriented approach and produced exactly the same results as our current approach. The second alternative approach focused on correlating gene expression with cytotoxicity, then finding SNPs associated with those genes, and evaluating the final SNPs with cytotoxicity. For SNP and gene-expression association, all approaches used Bonferroni correction with the number of transcript clusters in that particular analysis. Since the second alternative approach had fewer transcript clusters in the denominator (only gene expression that was significantly correlated with cisplatin IC_{50}), the *P* value cutoff was less stringent and resulted in a higher number of SNPs.

Although all 26 genes should be considered important, there are a number of genes with considerable evidence in the literature—for example, *DDIT4, NEK2,* and *MYC*. *DDIT4* is a known mediator of reactive-oxygen species generation.[20] The role of *DDIT4* in cell sensitivity to cisplatin has been suggested in the Affymetrix Human HG-U133A chip analysis in NT2/D1 (human embryonal carcinoma) cell lines after cisplatin treatment, with up-regulation of *DDIT4* gene expression after cisplatin treatment coincident with a 60% decrease in cell viability.[21]

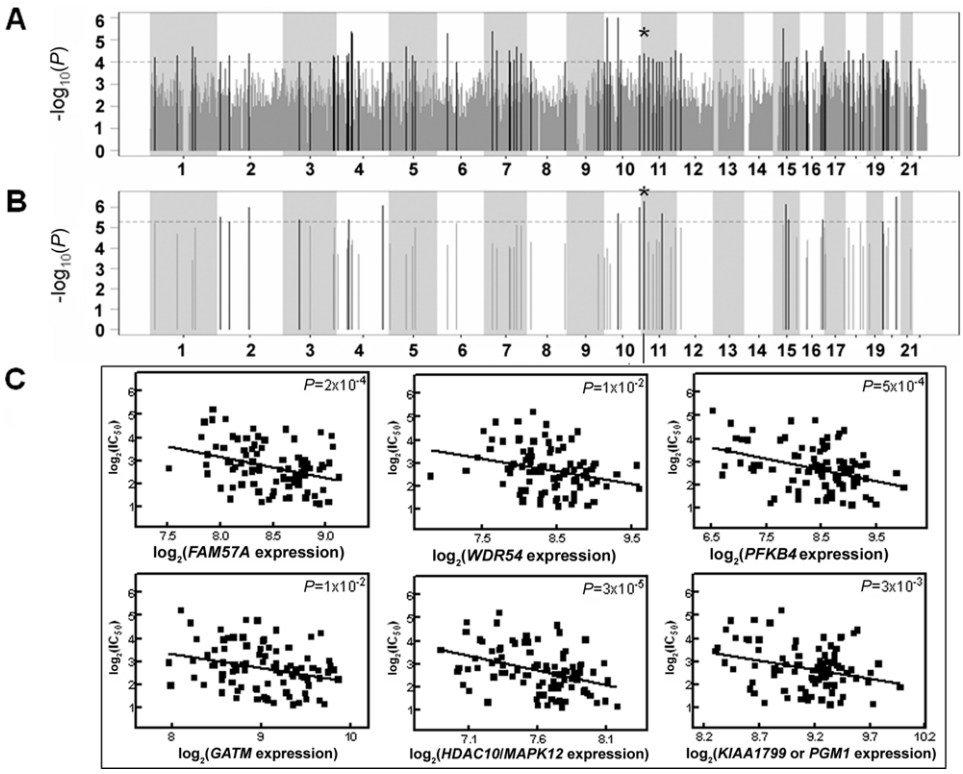

Figure 4. Relationship between SNP genotype, gene expression, and cisplatin IC_{50} in YRI population. *A,* Genomewide association between SNP genotypes and log_2-transformed cisplatin IC_{50} in YRI population. The *X*-axis represents the chromosomal location of SNPs. The *Y*-axis represents the statistical significance of association analysis. The dashed line indicates the significance cutoff ($P \leqslant 10^{-4}$). *B,* Association analysis between SNP genotypes and log_2-transformed gene expression in YRI population. The *X*-axis represents the SNP chromosomal location. The *Y*-axis represents the statisticial significance of association analysis. The dashed line indicates the significance cutoff (Bonferroni-corrected $P < .05$). *C,* Correlation between log_2-transformed cisplatin IC_{50} and log_2-transformed *FAM57A, WDR54, PFKFB4, GATM, HDAC10, MAPK12, KIAA1799,* and *PGM1* expression. *HDAC10* and *MAPK12* share the same transcript cluster ID on exon array, as do *KIAA1799* and *PGM1*. All six transcript clusters are significantly associated with one SNP genotype. This SNP, *rs12278731,* is labeled with an asterisk in panels A and B.

Furthermore, the higher *DDIT4* gene expression was also observed in the SQ20b (head and neck cancer) cell line after treatment with O^6-benzylguanine plus cisplatin, compared with cisplatin alone. This higher gene expression was concomitant with higher cisplatin-induced cytotoxicity, further suggesting that higher *DDIT4* expression level may lead to enhanced sensitivity to cisplatin.[22] Our study showed that the TT genotype of SNP *rs456998* was associated with higher *DDIT4* gene expression and higher sensitivity to cisplatin, in agreement with literature evidence.[22] Given the evidence of inducibility of this gene by cisplatin, we are currently evaluating *DDIT4* expression changes over time after treatment.

NEK2, another gene whose expression is strongly associated with the *rs456998* genotype, is required for the correct execution of mitosis.[23] Zhang et al. have shown that irradiation-induced DNA damage leads to inhibition of Plk1, which leads to subsequent inhibition of Nek2 activity and thus prevents centrosome separation in HeLa and U2OS cell lines.[24] The depletion of Nek2 leads to an apparent arrest in Hela cell proliferation and an increase in apoptosis, possibly as a result of mitotic errors.[25,26] Our current study showed that the TT genotype of SNP *rs456998* was associated with higher *NEK2* gene expression and higher sensitivity to cisplatin. Using the Affymetrix U133A microarray, we found that Nek2 expression patterns were significantly different between cisplatin-sensitive and cisplatin-resistant LCLs after 8 h of cisplatin treatment (S. Duan, W. Bleibel, C. Cheng, and M. E. Dolan, unpublished data).

Another interesting finding is the significant association of the genotype of SNP *rs8094647* with cisplatin IC_{50} ($P = 2 \times 10^{-5}$), as well as with the expression of the *MYC* gene ($P = 1 \times 10^{-6}$). The protein encoded by *MYC* is a multifunctional, nuclear phosphoprotein that plays a role in cell-cycle progression, apoptosis, and cellular transformation. It functions as a transcription factor that regulates transcription of specific target genes. Pretreatment with 10058-F4, a small-molecule c-Myc inhibitor, increased the chemosensitivity of HepG2 (hepatocellular carcinoma) cells to low-dose doxorubicin, 5-fluorouracil, and cisplatin.[27] We found that the GG genotype of *rs8094647* was

associated with higher *MYC* gene expression and lower cisplatin IC_{50}. Because it has been shown that cisplatin treatment down-regulates *MYC* gene expression at both the mRNA level (in NT2/D1 cells)[21] and protein level (in Hela cervical carcinoma cells),[28] we are currently investigating the relationship between sensitivity to cisplatin, SNP genotype, and *MYC* gene expression after cisplatin treatment.

Because of the relatively small sizes of HapMap samples, our approach focused on SNPs within or close to gene regions with fairly high allele frequencies, to increase the power to detect significant genetic variants associated with the cytotoxicity. Furthermore, the multivariate model used to test association between multiple SNPs and IC_{50} demonstrated the power of our approach. With a limited number of SNP genotypes (four, two, and four SNPs in the combined, CEU, and YRI populations, respectively), we were able to predict 27%, 29%, and 45% of the cisplatin IC_{50} variation in these LCLs. Although the full implications and biological significance of other genes and networks identified through our approach are not yet completely understood, these studies will likely help to direct clinical studies by providing a strong list of candidate genes on which to focus. They may serve as a platform for the further exploration of relevant mechanisms and may improve our understanding of the molecular basis of cisplatin-induced cytotoxicity. We are currently validating these findings in a set of unrelated CEPH cell lines.

One potential limitation of these cell lines is that EBV transformation could have an effect on drug sensitivity and/or expression profiles. Therefore, the model could miss some genes of importance because either they are not expressed in these cell lines or, upon transformation, the genes are down-regulated. Another important caveat of the model is the use of gene-expression analysis to narrow the number of associated genetic variants. The potential to miss genetic variants that affect cytotoxicity through effects on protein structure, activity, and/or degradation exists. However, the association test between cisplatin IC_{50} and genotype (current approach, first step) is inclusive of all genetic variants that act through these means (see the tab-delimited ASCII file of data set 1 [online only]). Furthermore, the dense genotyping within HapMap cell lines allows systematic, genomewide association analysis that would not be possible in other systems. We recognize that there likely are expression and posttranslational-modification differences in various tissues. Of the 26 genes we identified in LCLs, 18 were also expressed in human kidney tissue, which is one of the major sites of cisplatin-induced toxicity (GeneCards Web site). The genetic variants that are associated with cisplatin cytotoxicity within genes in human kidney are decent candidates to evaluate with patients that experience nephrotoxicity. Furthermore, the model can be applied to uncover the genetic signatures contributing to a wide range of cellular phenotypes.

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Web Resources

The accession number and URLs for data presented herein are as follows:

Affymetrix, http://www.affymetrix.com/products/arrays/exon_application.affx
The Coriell Institute for Medical Research, http://www.coriell.org/
GeneCards, http://www.genecards.org/
GEO, http://www.ncbi.nlm.nih.gov/geo/ (for all raw exon array data [accession number GSE7761])
Haploview software, http://www.broad.mit.edu/mpg/haploview/
International HapMap Database, http://www.hapmap.org/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for *NEK2, SHMT2, MYO5B, MYC, NRG3, BHLHB3, PFKFB4, GATM, HDAC10, MAPK12,* and *PGM1*)
PharmGKB Platinum Pathway, http://www.pharmgkb.org/search/pathway/platinum/platinum.jsp
QTDT software, http://www.sph.umich.edu/csg/abecasis/QTDT/

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