Integrating Cell-Based and Clinical Genome-Wide Studies to Identify Genetic Variants Contributing to Treatment Failure in Neuroblastoma Patients

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High-risk neuroblastoma is an aggressive malignancy, with high rates of treatment failure. We evaluated genetic variants associated with in vitro sensitivity to two derivatives of cyclophosphamide for association with clinical response in a separate replication cohort of neuroblastoma patients (n = 2,709). To determine sensitivity, lymphoblastoid cell lines (LCLs) were exposed to increasing concentrations of 4-hydroperoxycyclophosphamide (4HC; n = 422) and phosphoramid mustard (PM; n = 428). Genome-wide association studies were performed to identify single-nucleotide polymorphisms (SNPs) associated with sensitivity to 4HC and PM. SNPs consistently associated with LCL sensitivity were analyzed for associations with event-free survival (EFS) in patients. Two linked SNPs, rs9908694 and rs1453560, were found to be associated with (i) sensitivity to PM in LCLs across populations and (ii) EFS in all patients (P = 0.01) and within the high-risk subset (P = 0.05). Our study highlights the value of cell-based models to identify candidate variants that may predict response to treatment in patients with cancer.

A growing body of evidence suggests that common genetic variations within the host genome (as opposed to those within the tumor) affect treatment outcomes in patients with cancer who are treated with chemotherapy.6–8 Nonetheless, pharmacogenomic studies in oncology have been impeded by the large numbers of patients needed to achieve adequate power to detect associations in a population treated with multidrug regimens. Human cell–based models, in which genetic variation, gene expression, and response to a given drug (cytotoxicity, apoptosis, and cell cycle arrest) can be measured in large cohorts, have emerged as an important discovery platform for genetic variants associated with chemotherapeutic sensitivity and toxicity.9–11 The human lymphoblastoid cell line (LCL) repertoire generated by the International HapMap Project12 is a particularly useful cell-based resource because of its wealth of publicly available genotype information that can be used in genome–wide association studies (GWASs). The cell lines can be tested for genotype–phenotype associations under identical...
conditions, without any of the confounders encountered in vivo, and findings from the model can then serve as candidates for clinical validation.

Previously, our group has used this model to identify genetic variants associated with paclitaxel-induced peripheral neuropathy, cisplatin response in head and neck cancer, and carboplatin response in ovarian cancer. In this study, we evaluated active metabolites of the prodrug cyclophosphamide, an agent used to treat numerous malignancies, and a cornerstone of neuroblastoma treatment. 4-Hydroxycyclophosphamide is the active metabolite formed by oxidation of the parent drug, and it leads to the therapeutically beneficial DNA cross-linking agent phosphoramide mustard (PM). We sought to determine the role of genetic variants associated with in vitro sensitivity to two preactivated metabolites of cyclophosphamide—4-hydroperoxycyclophosphamide (4HC) and PM cyclohexylamine salt—in terms of both neuroblastoma risk group classification and response to therapy in a large cohort of children enrolled in a Children’s Oncology Group (COG) biology study, ANBL00B1. We hypothesized that genetic variants associated with cellular resistance to cyclophosphamide would also be associated with poorer event-free survival (EFS) in high-risk neuroblastoma patients.

RESULTS

Cellular phenotyping

Figure 1 illustrates our analytic approach. LCLs were phenotyped for sensitivity to 4HC and PM. Specifically, Caucasian of European origin (CEU) LCLs \((n = 163\) for 4HC; 164 for PM), Yoruba from Ibadan, Nigeria (YRI) LCLs (176 for 4HC; 177 for PM), and African-Americans of Southwestern US origin (ASW) LCLs \((n = 83)\) were exposed to increasing concentrations of 4HC and PM. Figure 2 illustrates the results of analysis of the pharmacologic phenotype for 4HC and PM by ethnic population. Comparisons between panels revealed the ASW population to be the most sensitive to the cytotoxic effects of 4HC, with a median area under the curve (AUC) significantly lower than that in the CEU \((P = 1 \times 10^{-4})\) and the YRI \((P < 1 \times 10^{-4})\) populations.

Figure 1 Analytic approach and cell-based genome-wide association results. (a) Analytic approach. Single-nucleotide polymorphisms with suggestive significance in the CEU lymphoblastoid cell line (LCL) population were validated in both the YRI and ASW LCL populations before final validation in patients with neuroblastoma. (b) Genome-wide association results of cellular sensitivity to 4-hydroperoxycyclophosphamide (4HC) and phosphoramide mustard (PM) in the CEU population. The Manhattan plot shows the results of genome-wide association studies of log-transformed area under the curve for 4HC and PM \((n = 163\) for 4HC; 164 for PM individual samples). ASW, African-Americans of Southwestern US origin; CEU, Caucasian of European origin; YRI, Yoruba from Ibadan, Nigeria.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

✓ High-risk neuroblastomas are clinically aggressive tumors that account for a disproportionate number of deaths.
✓ Identifying and avoiding the chemotherapeutic(s) that are ineffective in a multimodality regimen can prove difficult.

WHAT QUESTION DID THIS STUDY ADDRESS?

✓ We aimed to identify the genetic variants associated with sensitivity to cyclophosphamide in a human cell–based model and to evaluate whether these variants are also associated with EFS in a clinical study that included nearly 3,000 children with neuroblastoma.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE

✓ Polymorphisms in the IKZF3-ZPBP2 locus contribute to failure of neuroblastoma treatment, probably through resistance to phosphoramide mustard, the metabolite of cyclophosphamide that is thought to be responsible for its antitumor effect.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY AND THERAPEUTICS

✓ Our study highlights the value of cell-based models to complement clinical trials aimed at identifying and prioritizing genetic variants associated with nonresponse in rare diseases, such as neuroblastoma, for which large-scale GWASs of survival are difficult to replicate. Our approach could easily be adapted to other chemotherapies for a variety of malignancies.
Articles

Neuroblastoma cohort

Genotypes of 3,404 neuroblastoma patients enrolled in ANBL00B1 were used. After sample-based quality control filtering, as previously described,\textsuperscript{16} 2,709 patients formed the analytic cohort (Table 1).

GWAS of sensitivity to 4HC and PM in the CEU population

Figure 1b summarizes the results of the GWAS of sensitivity to 4HC and PM in the CEU population. No variant reached the genome-wide significant threshold of $5 \times 10^{-8}$ for either metabolite. Nevertheless, we observed that the top single-nucleotide polymorphisms (SNPs) were enriched for expression quantitative trait loci (empirical $P < 0.001$)\textsuperscript{17} and that limiting the association analysis to these expression quantitative trait loci improves our power to detect associations. Thus, the top SNPs from this analysis (see Supplementary Materials online) are enriched for SNPs that have been previously shown to be functionally important due to their association with gene expression.

Validation of CEU variants

No variant associated with sensitivity to 4HC in the CEU population could be validated in both the YRI/ASW populations and the neuroblastoma cohort. On the other hand, a variant, rs9908694, within intron 5 of the gene \textit{IKZF3}, was associated with sensitivity to PM in the CEU ($P = 5.62 \times 10^{-5}$), YRI ($P = 0.049$), ASW ($P = 0.05$), and high-risk neuroblastoma populations ($P = 9.67 \times 10^{-4}$, compared with patients with low- or intermediate-risk neuroblastoma). These results are summarized in Figure 3. The Supplementary Materials list the top population-specific SNP associations with the PM phenotype. The SNP rs9908694 is a common genetic variation in all panels examined here and has a much higher minor allele frequency (MAF) in the African panels (YRI (MAF = 0.31) or ASW (MAF = 0.16)) than in the CEU (MAF = 0.05) panel. The Supplementary Materials online show the SNP’s global allele frequency distribution, as represented in the Human Genome Diversity Project,

Table 1 Clinical characteristics of the analytic cohort.

| Clinical characteristic | No. (%) |
|-------------------------|---------|
| Age (months)            |         |
| <18                     | 1,285 (47.4) |
| >18                     | 1,424 (52.6) |
| Stage                   |         |
| 1, 2, 3, and 4s         | 1,459 (54.2) |
| 4                       | 1,233 (45.8) |
| Sex                     |         |
| Female                  | 1,235 (45.6) |
| Male                    | 1,474 (54.4) |
| MYCN status             |         |
| Not amplified           | 2,067 (76.3) |
| Amplified               | 481 (17.8) |
| Unknown                 | 161 (5.9) |
| Ploidy                  |         |
| Hyperdiploid            | 1,642 (60.6) |
| Diploid                 | 805 (29.7) |
| Unknown                 | 262 (9.7) |
| Histology               |         |
| Favorable               | 976 (36) |
| Unfavorable             | 837 (30.9) |
| Unknown                 | 262 (9.7) |
| Risk group              |         |
| Low                     | 960 (35.4) |
| Intermediate            | 537 (19.8) |
| High                    | 1,212 (44.7) |
| Self-reported race, ethnicity |         |
| White, not Hispanic     | 2,112 (80) |
| Black, not Hispanic     | 230 (8.5) |
| Other                   | 367 (13.5) |

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Figure 2  Interpopulation differences in sensitivity to 4-hydroperoxycyclophosphamide (4HC) and phosphoramido mustard (PM). The ASW population was more sensitive than the CEU ($^* P = 0.0001$) or the YRI populations ($^* P = 0.0001$). There were no differences in sensitivities to PM among the three HapMap panels. AUC, area under the curve; ASW, African-Americans of Southwestern US origin; CEU, Caucasian of European origin; YRI, Yoruba from Ibadan, Nigeria.

Figure 3  The Supplementary Materials list the top population-specific SNP associations with the PM phenotype. The SNP rs9908694 is a common genetic variation in all panels examined here and has a much higher minor allele frequency (MAF) in the African panels (YRI (MAF = 0.31) or ASW (MAF = 0.16)) than in the CEU (MAF = 0.05) panel. The Supplementary Materials online show the SNP’s global allele frequency distribution, as represented in the Human Genome Diversity Project,
Articles showing that the risk-conferring ancestral allele T has a substantially higher frequency in sub-Saharan African panels than in the remaining world populations examined. A chromosome 3 SNP, rs9863764, intronic to glutamate receptor metabotropic 7 (GRM7), was associated with sensitivity to PM in the CEU population ($P = 8.08 \times 10^{-5}$) and showed, at best, only very modest association with EFS ($P = 0.09$) but was not associated with AUC in the YRI ($P = 0.21$) or ASW ($P = 0.96$) populations.

Impact of rs9908694 on neuroblastoma EFS

In Kaplan–Meier survival analyses, rs9908694 was associated with EFS in both the entire neuroblastoma cohort ($P = 0.01$) and the high-risk subpopulation ($P = 0.05$), with the proportion of African ancestry used as a covariate. Risk-allele frequency in the neuroblastoma cohort was 5.1%. Of note, patients with the risk allele, which confers resistance to PM, had less favorable EFS (Figure 4).

Enrichment of SNPs associated with outcome among SNPs associated with PM sensitivity

We evaluated whether SNPs in the CEU population associated with sensitivity to PM (at the $P$ value threshold $\leq 1 \times 10^{-4}$) harbor SNPs associated with EFS in neuroblastoma patients, and we found a significant excess of SNPs relative to expectation (see Methods and Supplementary Materials online).

Functional annotation

Within the SCANdb database, rs9908694 was associated with expression of seven transcripts in YRI LCLs (Figure 5): ORMDL3 ($P = 1 \times 10^{-5}$), DDIT3 ($P = 5 \times 10^{-5}$), NEU3 ($P = 6 \times 10^{-5}$), SLC38A2 ($P = 6 \times 10^{-5}$), MYC ($P = 7 \times 10^{-5}$), IGLV6-57 ($P = 1 \times 10^{-4}$), and ASNS ($P = 1 \times 10^{-4}$). In addition, rs9908694 was associated with the expression of its host gene, IKZF3 ($P = 0.03$).

The SNP is a strong enhancer, as derived from a hidden Markov model study that models the combinatorial patterns of observed histone modifications, and shows a chromatin immunoprecipitation sequencing (ChIP-Seq) peak on the basis of histone H3 acetyl K27 (H3K27ac) and histone H3 mono methyl K4 (H3K4me1) data (Broad ENCODE group) in the CEU cell line GM12878. The SNP is in perfect linkage disequilibrium ($r^2 = 1$, in the CEU, YRI, and ASW populations) with a SNP rs1453560 (located 1 kb 5' of ZPB2) that is located in a DNase hypersensitive site in a variety of cell types, including several YRI cell lines (GM18507, GM19238, GM19239, and GM19240), and is highly conserved (from Genomic Evolutionary Rate Profiling and SiPhy scores).

Figure 3 Sensitivity to phosphoramid mustard (PM) by rs9908694 genotype. The T allele of rs9908694, within intron 5 of the gene IKZF3, is associated with sensitivity to PM in the CEU ($P = 5.62 \times 10^{-5}$), YRI ($P = 0.049$), and ASW ($P = 0.05$) populations of lymphoblastoid cell lines. AUC, area under the curve; ASW, African-Americans of Southwestern US origin; CEU, Caucasian of European origin; YRI, Yoruba from Ibadan, Nigeria.

Figure 4 Event-free survival (EFS) by rs9908694 genotype. The T allele, which confers resistance to phosphoramid mustard (PM) in the lymphoblastoid cell line model, is associated with inferior EFS in the entire neuroblastoma cohort ($P = 0.01$) and within the subset of high-risk neuroblastoma patients ($P = 0.05$).
In particular, rs1453560 is associated with sensitivity to PM in the CEU (\(P = 5.62 \times 10^{-5}\)), YRI (\(P = 0.05\)), and ASW (\(P = 0.05\)) populations, as well as in the high-risk neuroblastoma group (\(P = 9.67 \times 10^{-4}\); \(P = 0.05\)). Furthermore, rs1453560 is associated with EFS in both the entire neuroblastoma cohort (\(P = 0.01\)) and the high-risk subpopulation (\(P = 0.05\)).

**Effect of IKZF3 siRNA knockdown**

Use of small interfering RNA (siRNA) against IKZF3 messenger RNA resulted in a significant decrease in host gene expression at all measured time points (\(P = 4.66 \times 10^{-11}\), \(P = 1 \times 10^{-13}\), and \(6.25 \times 10^{-13}\) for 5, 29, and 53 h, respectively). In addition, the expression of several of the predicted expression quantitative trait locus targets of our IKZF3 SNP was altered by IKZF3 siRNA. As outlined in Figure 6, significant alterations, compared with nontargeting siRNA, were seen at 53 h posttransfection for ASNS (\(P = 0.03\)) and IGLV6-57 (\(P = 4.84 \times 10^{-6}\)) and at 29 h posttransfection for MYC (\(P = 0.02\)).

**DISCUSSION**

In this study, we used the International HapMap CEU LCL resource to identify variants associated with sensitivity to an activated form of cyclophosphamide and its metabolite PM. A variant associated with in vitro sensitivity to PM (rs9908694) in CEU LCLs was replicated across two other LCL panels (\(P \leq 0.05\)). This SNP was associated with both high-risk neuroblastoma status and with EFS in a cohort of 2,709 children with neuroblastoma. Furthermore, in children with high-risk neuroblastoma, those with the rs9908694 risk allele had poorer EFS than patients homozygous for the nonrisk allele, suggesting that the SNP’s impact on survival is independent of neuroblastoma risk group classification. This variant was associated with the expression of the host gene, IKZF3 (\(P = 0.03\)). YRI, Yoruba from Ibadan, Nigeria.

![Figure 5](image-url)  
**Figure 5** Association between rs9908694 genotype and expression, measured on the Affymetrix GeneChip Human Exon 1.0ST Array in lymphoblastoid cell lines of YRI ancestry. The genotypes are plotted against baseline gene expression of ORMDL3 (\(P = 1 \times 10^{-5}\)), DDIT3 (\(P = 5 \times 10^{-5}\)), NEU3 (\(P = 6 \times 10^{-5}\)), SLC38A2 (\(P = 6 \times 10^{-5}\)), MYC (\(P = 7 \times 10^{-5}\)), IGLV6-57 (\(P = 1 \times 10^{-5}\)), and ASNS (\(P = 1 \times 10^{-5}\)). In addition, rs9908694 was associated with the expression of its host gene, IKZF3 (\(P = 0.03\)). YRI, Yoruba from Ibadan, Nigeria.
Cyclophosphamide is an integral component of therapies targeting both intermediate- and high-risk neuroblastoma and is also used in the treatment of many other cancers. Cyclophosphamide is a prodrug converted by a number of cytochrome P450 enzymes to 4-hydroxycyclophosphamide. 4-Hydroxycyclophosphamide spontaneously (nonenzymatically) leads to the formation of acrolein, a urotoxic metabolite with no known antitumor properties, and the active alkylating compound PM. 4HC is a synthetic, preactivated analog of cyclophosphamide, which, under aqueous conditions, is spontaneously converted to 4-hydroxycyclophosphamide. This metabolite then provides for the formation of PM through the usual metabolic pathway of cyclophosphamide. Because 4HC, like cyclophosphamide, produces PM and acrolein simultaneously, we chose to include authentic PM in our experiments. We expected that this would allow for an understanding of the germ-line genetic variants associated with the toxicity and efficacy of the parent cyclophosphamide (via 4HC), distinct from those associated with the ultimate metabolite of therapeutic consequence (PM). In addition, genetic variants within enzymes responsible for the bioactivation, metabolism, and clearance of cyclophosphamide were not able to explain the interindividual variability in the clearance of cyclophosphamide or 4-hydroxycyclophosphamide, suggesting that variants outside of the established pharmacokinetic pathway may be responsible for this interindividual variability. Our inability to validate variants associated with in vitro sensitivity to 4HC within the neuroblastoma survival analysis may be due to the fact that this metabolite generates both toxic and therapeutic compounds, whereas PM only contributes to the therapeutic effect of cyclophosphamide and is therefore a purer representation of response to therapy.

The SNP rs9908694 lies within intron 5 of IKZF3 on chromosome 17q21, and unbalanced gains of this chromosomal region are found frequently in clinically aggressive neuroblastoma. IKZF3 is one of the five genes in the Ikaros family of zinc finger DNA-binding transcription factors important in the regulation of lymphocyte differentiation. Although the host gene (IKZF3) for one of our predictive SNPs has not been previously implicated in either neuroblastoma pathogenesis or cyclophosphamide response, the expression of the host gene and several other transcripts appears to be affected by the presence of the (expression quantitative trait locus) rs9908694 risk allele, which may provide insight into the mechanism of this SNP’s negative impact on EFS seen in our cohort. Interestingly, three of the seven genes targeted by rs9908694—DDIT3, ORMDL3, and ASNS—are thought to be involved in the stress response of the endoplasmic reticulum, a process that is often dysregulated in human cancers including neuroblastoma, leading to inherent resistance to cell death. The SNP is in perfect linkage disequilibrium in all populations examined here, with a SNP, rs1453560 (1 kb upstream of ZPBP2), that is in a DNAse hypersensitive site, which may provide insights for future studies on the role of this locus in treatment failure.

Integrative epidemiology studies have previously linked disease susceptibility variants to those associated with treatment response, and our findings may serve as an additional example of this concept. The IKZF3-ZPBP2 locus (importantly, the SNP rs1453560 from our study) has been implicated, in a recent large-scale multiethnic study, as a susceptibility locus for...
for systemic lupus erythematosus, which is often treated with cyclophosphamide. Furthermore, the SNP rs9908694 has been found to be nominally associated with glycosylated hemoglobin in a genome-wide study in a British population. Aberrant glycosylation occurs in human cancers and has been implicated in neuroblastoma.

High-risk neuroblastomas are clinically aggressive tumors that account for a disproportionate amount of pediatric deaths due to cancer. Identifying patients at risk for failure of treatment may lead to improvements in treatment delivery for patients with cancer who have little chance of cure. However, identifying and avoiding the component(s) responsible for failure of treatment in multimodality regimens can prove difficult. The methods outlined here support the view that polymorphisms in the IKZF3-ZPBP2 locus contribute to failure of treatment for neuroblastoma, probably through resistance to cyclophosphamide and its metabolites.

Our study highlights the value of cell-based models in the identification of candidate variants that may predict response to treatment in patients with cancer. Drawbacks of this approach include the weight of validation in other ethnic populations with markedly different linkage disequilibrium patterns. Another drawback is that patients in this study were enrolled in a biobanking protocol separately from an available treatment protocol, and most, but not all, patients enrolled in the protocol were treated as part of a Children’s Oncology Group clinical trial. Our approach could be easily adapted to other cytotoxic chemotherapies for a variety of malignancies. Clinical validation of the most highly significant SNPs from cell-based models may provide important insights into the genetic architecture of human response to chemotherapy and may inform the interpretation of results from past clinical studies and the design of future studies. Prospective validation of the variant that we identified and its impact on survival will be needed before clinical implementation.

**METHODS**

**Drugs and lymphoblastoid cell lines.** Cyclophosphamide is a prodruk requiring activation to 4-hydroxycyclophosphamide by the cytochrome P450 system; however, LCLs show low expression of the P450 enzymes that are required for this oxidation. 4HC serves as a synthetic equivalent of 4-hydroxycyclophosphamide; under aqueous conditions, it is spontaneously converted to 4-hydroxycyclophosphamide and, ultimately, PM. The synthesis of 4HC has been previously described. Phosphoramide mustard, as the cyclohexylammonium salt, was a gift from the Drug Synthesis and Chemistry Group at the National Cancer Institute.

HapMap LCLs derived from (i) Caucasian individuals (n = 163 for 4HC and n = 164 for PM) from Utah, United States, with northern/western European ancestry (CEU), (ii) Yoruba individuals (n = 176 for 4HC and 177 for PM) from Ibadan, Nigeria (YRI), and (iii) African-American individuals (n = 83 for both 4HC and PM) from the southwestern United States (ASW) were used for drug sensitivity phenotyping. LCLs were purchased from the Coriell Institute for Medical Research (Camden, NJ) and cultured in RPMI 1640 media (Cellgro, Herndon, VA) containing 15% heat-inactivated bovine growth serum (HyClone, Logan, UT) and 20 mmol/L1-glutamine.

**Cellular phenotyping.** Cell lines were diluted three times per week at a concentration of 300,000–350,000 cells/ml and incubated at 37 °C with 5% CO₂/95% humidified air. Cell growth inhibition for a given cell line was evaluated at concentrations of 0, 1, 5, 10, 20, and 40 µmol/l for 4HC and 0, 5, 10, 25, 50, 100, and 200 µmol/l for PM in separate experiments. Aqueous solutions of 4HC and PM are unstable; thus, they were made fresh as needed, kept cool (ice bath), and used as quickly as possible (maximum lag time between dissolution and use: 30 min). Cell lines in the exponential growth phase with >85% viability (Vi-Cell XR viability analyzer, Beckman Coulter, Fullerton, CA) were plated in triplicate in 96-well plates (Corning, Corning, NY) 24 h before drug exposure. LCLs were exposed to drug suspended in phosphate-buffered saline (Life Technologies, Carlsbad, CA) for 72 h. Cell growth inhibition was measured by the Alamar Blue assay (Life Technologies) at wavelengths of 570 and 600 nm (Synergy-HT multideck plate reader, BioTek, Winooski, VT). The AUC was determined for each LCL through the trapezoidal rule and log-transformed for further data analysis. Cell lines were excluded from analysis for poor growth in culture or failure to achieve a reproducible cellular sensitivity to drug.

**GWAS of cellular sensitivity to 4HC and PM.** CEU genotypes (n = 180) were downloaded from the HapMap Consortium release 27. Log-transformed AUC values were used as phenotype. A GWAS was performed in CEU LCLs to estimate sensitivity to 4HC and PM (separately; Figure 1b). More than 2 million SNPs (MAF > 5%, no Mendelian errors, and in Hardy–Weinberg equilibrium) were tested for association with either 4HC or PM.

We conducted GWASs of sensitivity to 4HC and PM (separately) in the two African panels—YRI and ASW. Because ASW contains samples of recent admixture, we used the proportion of African ancestry, derived from principal component analysis, in these samples as a covariate in the association analysis. Those SNPs that achieved a nominal level of significance (defined as P ≤ 1 × 10⁻⁵) in the CEU population were tested for association in the YRI population (P < 0.05 and concordant direction). Those SNPs that passed this “validation” step were also tested for association in the ASW population. SNPs that survived this multistep validation analysis in independent sets of LCLs were then taken forward for validation in the clinical data set.

**Neuroblastoma patient cohort and quality control of patient data.** After the participating institutions obtained the approval of their institutional review boards and after informed consent was obtained, children diagnosed with neuroblastoma, ganglioneuroblastoma, or ganglioneuroma (maturing type) were enrolled in COG ANBL00B1 (NCT00904241) between 2001 and 2009. ANBL00B1 was a neuroblastoma biology registry, and selection of therapy was at the discretion of treating physicians and families. The majority of patients would have also been enrolled in a therapeutic clinical trial through the COG. Patients with available genotype, risk group classification, and outcome data formed the analytic cohort. Methods to confirm the diagnosis, assignment of stage, analysis of tumor biology (ploidy, MYCN amplification, and histology), and assignment of self-reported race have been previously described.

DNA samples from patients enrolled in COG ANBL00B1 were genotyped using three Illumina (San Diego, CA) platforms: 550v1, 550v3, and Human610-Quad, as previously described. Included in this analysis are those SNPs that were genotyped on all three platforms, had call rates >95%, and had MAFs >5%.

The sample-based quality control pipeline for this cohort has previously been described. Briefly, extensive quality control analyses were performed on the genotype data, including detection of sex incompatibilities, misspecified relationships, and duplications. Call rates were estimated by individual and by SNP in order to determine average heterozygosity (across all SNPs) for each sample and to evaluate genotype distributions for each SNP in relation to expected Hardy–Weinberg equilibrium.

**Association of PM-sensitive variants with high-risk neuroblastoma phenotype and EFS.** SNPs that achieved significance in the CEU population and were validated in the YRI and ASW populations were tested.
for association \((P < 0.05)\) with the high-risk neuroblastoma phenotype (Figure 1). The risk allele in the LCL model was evaluated for its (additive) effect on high-risk disease in patients. Because the patient cohort includes both Caucasians and African Americans, we used the proportion of African ancestry in the association analysis, as previously described.\(^{16}\) SNPs that achieved significance in both the cell-based model and the neuroblastoma cohort were tested for their impact on EFS among all patients in the analytic cohort as well as in the subset of high-risk patients alone. For each such SNP, log-rank comparison of EFS by SNP genotype was performed.

Enrichment analyses. We conducted a permutation resampling analysis to test for enrichment of 4HC and PM cytotoxicity-associated SNPs identified in the LCL model among SNPs associated with the high-risk phenotype in the neuroblastoma cohort. Using a previously described method,\(^{34}\) the risk group classification (high-risk vs. non–high-risk phenotype) was randomly shuffled while keeping the genotype data fixed, thereby preserving linkage disequilibrium. Based on this permuted replicate, logistic regression analyses were conducted for all the SNPs. This process was repeated 1,000 times. For each of the 1,000 replicates, the number of SNPs with \(P \leq 0.05\) in the patient data and \(P \leq 0.0001\) in the LCL data, with concordant direction of effect, was calculated. The distribution of the overlap count from the permutations was compared with the distribution of the actual overlap count to generate an empirical \(P\) value. The significance of the excess of high-risk neuroblastoma–associated SNPs among cellular sensitivity–associated SNPs was calculated as the proportion of permutations in which the number of actual overlap SNPs that matched or exceeded the observed overlap SNP count. We considered the most highly ranked SNPs in the LCL data \((P \leq 0.0001)\) and generated a quantile–quantile plot from their association with high-risk neuroblastoma.

Impact of validated variant on LCL gene expression. Baseline gene expression was evaluated in 89 YRI and 87 CEU LCLs using the Affymetrix GeneChip Human Exon 1.0ST Array (Affymetrix, Santa Clara, CA), as previously described.\(^{45}\) SNPs validated across the cell-based model were associated with drug sensitivity in the cell-based model and with EFS in patients, we downloaded and used the public ENCODE data assayed in a variety of cell types (including the CEU cell line GM12878 and the YRI cell lines GM18507, GM19238, GM19239, and GM19240) to determine the chromatin states of potentially regulatory regions and to identify DNAse hypersensitivity sites.\(^{19}\)

siRNA knockdown of IKZF3. The LCLs GM18856 and GM19093 were counted (Vi-Cell, Beckman Coulter) and diluted to \(5 \times 10^5\) cells/ml 24 h before nucleofection. Cells (viability \(>85\%\)) were nucleofected using Lonza’s Cell Line 96-well Nucleofector Kit SF (Lonza, Basel, Switzerland). The reaction was performed according to the manufacturer’s protocol using \(1 \times 10^6\) cells per well and 2,000 nmol/l final concentration of AllStars Negative Control siRNA (Qiagen, Valencia, CA) or a pool of IKZF3 FlexiTube siRNA (Hs_IKZF3_1, Hs_IKZF3_2, Hs_IKZF3_3, and Hs_ZNFN1A3_5; Qiagen). The cells were nucleofected using the DN-100 program on an Amaxa Nucleofector 96-well shuttle (Lonza). After a 10-min rest, 85 \(\mu\)l prewarmed medium was added to each transfected well, and cells were allowed to rest for an additional 5 min. Cells were plated for messenger RNA pelleting in 24-well flat-bottom plates. Cells were harvested at 5, 29, and 53 h after nucleofection, washed in ice-cold phosphate-buffered saline, and centrifuged to remove phosphate-buffered saline. All pellets were stored at \(-80^\circ\)C until RNA isolation.

Quantitative real-time PCR. See Supplementary Materials.

Statistical analysis of IKZF3 knockdown. Significance across multiple cell lines for the effect of IKZF3 knockdown on the target genes’ expression was evaluated using a mixed-effects model combining the two cell lines and using cell line ID as random effect. Log2 relative expression for a given time and gene was tested to find whether it was different from zero. The R statistical software and the lme4 package were used to fit the mixed-effects model.\(^{46,47}\)

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/cpt

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AUTHOR CONTRIBUTIONS

N.J.C., S.L.C., M.E.D., N.P., and E.R.G. wrote the manuscript. N.J.C., S.L.C., and M.E.D. designed the research. S.J.D., J.M.M., N.P., E.R.G., N.A., and J.M. performed the research. S.J.D., J.M.M., N.P., E.R.G., A.L.S., A.K., H.K.I., and W.B.L. analyzed the data. S.M.L. contributed new reagents/analytical tools.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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