Heritability and Linkage Analysis of Sensitivity to Cisplatin-Induced Cytotoxicity

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ABSTRACT

Little is known about the genetic determinants explaining variation in sensitivity to chemotherapeutic cytotoxicity. We characterized the degree of cisplatin sensitivity, using lymphoblastoid cell lines derived from 10 Centre d’Etude du Polymorphisme Humain pedigrees. We estimated the heritability for susceptibility to cisplatin-induced cytotoxicity to be ~0.47; therefore, sensitivity to the cytotoxic effects of cisplatin is under appreciable genetic influence. Linkage analysis was performed, and the strongest signal (lod score, 2.16; empirical P = 0.0005) was found on chromosome 1 at 44 cM. Susceptibility to cisplatin-induced cytotoxicity is likely due to multiple loci, with low locus-specific heritability contributing to the trait. These data show the power of using large pedigrees that have been extensively genotyped for evaluating the genetic contribution to sensitivity to cell growth inhibition by anticancer agents.

INTRODUCTION

A long-term goal of pharmacogenomics research is the design of an individualized therapy based on the genomic sequence of the patient. Such an approach is particularly needed in cancer therapy because most chemotherapeutic drugs affect both tumor and normal cells, are ineffective in many patients, and exhibit serious, at times life-threatening, side-effects. Previous efforts to use genetic information to predict drug sensitivity have focused on a single candidate gene or pathway. Studying the genetic contribution to chemosensitivity in the clinic is challenging because drug responses reflect not only properties intrinsic to the target cell but also host metabolic properties. In addition, phenotypic studies on healthy volunteers are not ethical because of the toxicity associated with chemotherapy. Here we describe an approach that offers the advantage that no a priori assumptions are made about candidate genes to determine the contribution of genetic variables to susceptibility of cells to chemotherapy-induced cytotoxicity. By modeling chemosensitivity in EBV-transformed B-lymphoblastoid cell lines derived from large reference Centre d’Etude du Polymorphisme Humain (CEPH) pedigrees, we were able to dissect the genetic contribution of cell sensitivity to cisplatin-induced cytotoxicity by shielding it from variables that exist in vivo. These cell lines allow for the measurement of in vitro cytotoxicity while providing a considerable advantage in that extensive genotype data are available in the CEPH databases. The present study describes a novel strategy for testing the hypothesis that there are genetic determinants of cellular susceptibility to cisplatin.

MATERIALS AND METHODS

Cell Lines. Lymphoblastoid cell lines derived from the 10 Caucasian Utah CEPH families (1331, 1332, 1333, 1346, 1347, 1362, 1408, 1413, 1416, and 1423) were purchased from Coriell Institute for Medical Research (Camden, NJ). Lymphoblastoid cell lines were cultured in RPMI 1640 containing 15% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 1% penicillin/streptomycin, and 20 μM l-glutamine and maintained in a humidified incubator at 37°C and 5% CO2. Medium and components were purchased from Cellgro (Herndon, VA).

Cell Cytotoxicity Assay. Cells (2 ml) from exponentially growing cultures (~95% viable confirmed by trypan blue dye exclusion) were plated at a density of 500,000 cells/ml in 12-well plates for 24 h. Cells were then treated with vehicle or increasing concentrations of cisplatin for 48 or 72 h in the absence of penicillin/streptomycin. Cisplatin was purchased from Sigma Chemical Co. (St. Louis, MO) and prepared in DMSO immediately before use. Control and treated cells were exposed to the same amount of DMSO (0.1%). Cells were harvested, washed with PBS, stained with 10 μl of 500 μg/ml propidium iodide, and analyzed using a FACScan flow cytometer (Becton Dickinson) with the CellQuest program (Becton Dickinson) and FlowJo (Tree Star Inc). Cell death was determined by a propidium iodide intensity >100. Four families (60 individuals) were initially phenotyped to estimate heritability after treatment with 5, 10, or 20 μM cisplatin for 48 and 72 h. Subsequently, six additional families (147 total individuals) were phenotyped in efforts to increase the power for genetic linkage analysis. Lower concentrations (0, 1, 2.5, 5, and 10 μM) at 48 h were used to capture the phenotypic effect in sensitive cell lines.

Heritability Analysis. Heritability analysis was done using Sequential Oligogenic Linkage Analysis Routines (SOLAR) computer software (1). SOLAR uses likelihood ratio tests to evaluate heritability by comparing a purely polygenic model with a sporadic model in the case of testing heritability. This method also allows for the testing of covariates in all of the models.

Error Checking. Error checking for Mendelian incompatibility, misspecified relationships, and unlikely recombinations was done with a web-based platform for linkage analysis developed by our group. The web-based platform integrates and formats data (pedigree, genotype, and phenotype); executes error checking by use of PedCheck (2) to detect genotypic incompatibilities, PREST (3) to detect relationship misspecifications, and MERLIN (4) to detect unlikely recombinants before linkage analysis; and is enabled to run linkage analysis on multiple platforms, including MERLIN and GENEHUNTER. From the combined pool of genotyped markers, 1784 nonredundant markers yielding a very dense genetic map with highly heterozygous markers (heterozygosity: 1% at <0.70, 7% at 0.7–0.8, 28% at 0.8–0.9, and 64% at 0.9–1) were used.

Linkage Analysis. SOLAR was used to perform linkage analysis, using variance components that compare the likelihood of a model that permits a particular locus (possible quantitative trait locus) to account for additive genetic variance, with a residual polygenic component, with a second, purely polygenic model. This method is the most powerful when the trait is normally distributed. To test whether there was an increase in false positives due to the distribution of the data, a completely informative marker, unlinked to the phenotype, was simulated, and the identity-by-descent (measure of allele sharing between relative pairs) was calculated. For each replicate, the variance components lod score was calculated, using the observed phenotypes. We simulated 20,000 replicates. Empirical P values were calculated by counting the number of replicates that equaled or exceeded the observed lod score and dividing that number by the total number of replicates. Merlin was used to perform nonparametric linkage analysis (NPL). This method does not require that the data be normally distributed. The genotypic data were downloaded from the CEPH database 6 and the Marshfield map database 7 and error-checked using the above-described methods. Lod P values for both analyses were calculated using the methods in Nyholt (5).

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6 http://www.cepbh.fr/cephpdb/
7 http://research.marshfieldclinic.org/genetics

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RESULTS

Sensitivity of Cells to Cisplatin. Using propidium iodide exclusion as a measure of cell viability, we evaluated the effect of cisplatin on the growth of individual lymphoblastoid cell lines representing four three-generation CEPH/Utah pedigrees (1333, 1347, 1416, and 1423). We used cell lines that were in logarithmic growth for 2 weeks to limit the variation due to growth conditions. Cells in exponential growth were exposed to 0, 5, 10, and 20 μM for 48 and 72 h (Fig. 1A; data not shown for 72 h). On the basis of the sensitivity of cells to 5 μM cisplatin (e.g., family 1347 had an average of 37% survival), we readjusted the concentrations under study by adding lower concentrations (1 and 2.5 μM) and removing 20 μM cisplatin for the remaining six families (1331, 1332, 1346, 1362, 1408, and 1413). Thus, the concentrations evaluated on the additional six families were 1, 2.5, 5, and 10 μM for 48 h (Fig. 1B). A total of 147 cell lines were evaluated, giving mean (±SD) percentages of live cells after 48 h exposure to 5 and 10 μM cisplatin of 48 ± 11 and 33 ± 10%, respectively. The population variance was 87 and 73% for 5 and 10 μM cisplatin at 48 h, respectively. Frequency distribution plots of the phenotype revealed a normal distribution (Shapiro–Wilk, P < 1.0) at 5 μM, but the distribution at 10 μM was not normally distributed (Shapiro–Wilk, P < 0.01; Fig. 2).

Heritability Estimate. Heritability was calculated by comparing the covariance of the trait among individuals with the overall variance of the trait. Before the analysis, the degree of heritability was unknown. We therefore picked four families that were estimated to have 80% power to detect 75% heritability. We found significant heritability with these data (heritability range, 51–68%; P = 0.002–0.000002) for each time point and concentration. To improve the power of linkage analysis, six additional CEPH families comprising three generations were chosen. The 10 families gave us 93% power to detect heritability >50%. The heritability estimate for cell lines derived from 10 families (147 individuals) exposed to 5 and 10 μM cisplatin for 48 h was 0.38 (P = 0.00003) and 0.47 (P = 0.000002), respectively (Table 1).

Error Checking. We found 12,011 incompatibilities in 1674 markers (all from the Marshfield database) and zeroed them out. Unlikely recombinants, usually due to genotyping errors, included 324 markers on 21 chromosomes, and the affected genotypes were zeroed out. There was an opposite-sex sibling pair in 1416 that shared both alleles for 90% of markers. There was neither evidence of inbreeding from the other 8 siblings nor evidence of increased allele sharing between the parents. One member of the sibling pair had a much smaller number of markers genotyped than the other members of the family and did not have sufficient X-chromosome markers that gender could be determined unambiguously. It was concluded that...
there was a problem with the genotypes from this sibling; therefore, the sibling was excluded from the analysis. All of the other relationships checked with PREST were consistent with what was specified in the pedigree files.

**Linkage Analysis.** We anticipated that specific chromosomal regions are likely to harbor polymorphic candidate genes responsible for sensitivity to cisplatin-induced cytotoxicity; we therefore performed linkage analysis to correlate similarity of phenotype between relative pairs with marker allele sharing (Fig. 3). Two methods were chosen for linkage analysis: variance components analysis (VCA; genetic variation due to linked loci, used when data are normally distributed and unselected) and nonparametric linkage analysis (NPL; robust to nonnormality; less powerful than VCA for normal distributions). The phenotype with the higher heritability, dose 10 μM at 48 h, was used for the analysis. Linkage analysis on the 10 families indicated that the most significant findings were on chromosome 1 at 44 cM (VCA, lod score, 2.16, lod \( P = 0.0008 \); empirical \( P = 0.0005 \); NPL, lod score, 1.37, lod \( P = 0.006 \)) and chromosome 12 at 147 cM (VCA, lod score, 2.49, lod \( P = 0.07 \), empirical \( P = 0.054 \); NPL, lod score, 1.90, lod \( P = 0.002 \)). Lod scores of 2.16 and 1.9 are expected to occur 1.1 and 1.8 times by chance, respectively, in a single genome scan.

**DISCUSSION**

Many genes are likely to influence the toxicity associated with chemotherapy. This report describes the use of CEPH pedigrees to demonstrate that half of the variance associated with cisplatin-induced cytotoxicity is attributable to genetic influence. None of our results exceeded criteria for genome-wide significance (6) and therefore may be chance findings. The inability to identify one or more loci with lod scores significant at a genome-wide level is likely due to multiple loci contributing to the trait and the low power to detect QTLs of modest heritability and may require significantly more families to detect linkage than were studied in this sample. The long-term goal of these studies is to identify polymorphisms responsible for sensitivity to chemotherapeutic cytotoxicity, ultimately leading to the identification of patients at greater risk for their associated toxicities. Because the current sample appears to have low power to detect those polymorphisms, we plan on studying a significantly larger sample in the future to either confirm the findings reported here or to identify new regions that this sample did not have the power to detect.

The two linkage analyses highlight different chromosomal regions that may reflect false positives or are related to the different distributional assumptions of each test. Although the toxicity of the 10 μM cisplatin dose was not normally distributed, simulations demonstrate that the false-positive rate was not increased in this analysis. A SOLAR analysis of the toxicity of the 5 μM cisplatin dose, which was consistent with a normal distribution but of lower heritability, gave a peak lod score of 0.85 (lod \( P = 0.02 \)) in the same region.

There have been limited studies evaluating the genetic contribution to cellular effects caused by chemotherapeutic or carcinogenic damage. Genetic influences accounted for 75% of the total variance in interindividual susceptibility to bleomycin-induced chromatid breaks (7). Genetic factors were responsible for interindividual variations in aryl hydrocarbon hydroxylase induction by 3-methylcholanthrene as suggested by an index of heritability of 0.8 (8, 9). Lymphoblastoid cell lines from both Wilms’ tumor patients and their first-degree relatives showed increased sensitivity to the cross-linking agent mitomycin C (10). Cheung et al (11) recently demonstrated familial aggregation of expression phenotype, suggesting a genetic contribution to polymorphic variation in the level of gene expression. Therefore, polymorphic variation in expression levels of candidate genes important in sensitivity to growth inhibition by cisplatin could be responsible for our findings.

Cisplatin is effective against a wide range of cancers. In particular, cisplatin in combination with etoposide and bleomycin is considered a curative treatment for testicular cancer and is beneficial in combination regimens for ovarian cancer and cancers of the bladder, head and neck, endometrium, esophagus, and lung (12–15). Our data provide the first step in identifying heritable genes that put patients at risk for cisplatin-induced cytotoxicity.
GENETIC CONTRIBUTION TO CISPLATIN CYTOTOXICITY

Methods such as bioinformatics, functional genomics, and high-throughput screening approaches to significantly increase the number of families that can be evaluated will be required to elucidate the heritable genetic factors that contribute to this phenotype. Identifying genes that are important in sensitivity to chemotherapy will provide us with a potential target or targets for sensitizing human cancer cells to chemotherapy and will help predict whether an individual will experience severe toxicity associated with chemotherapy. Additionally, as long as a phenotype can be measured in these lymphoblastoid cell lines derived from multigeneration families, this model can be used for studying heritable genetic factors important in pharmacogenetics.

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