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Identification of genetic variants associated with capecitabine-induced hand-foot syndrome through integration of patient and cell-line genomic analyses

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Abstract

Objective—A primary challenge in identifying replicable pharmacogenomic markers from clinical genome wide association study (GWAS) trials in oncology is the difficulty of performing a second large clinical trial with the same drugs and dosage regimen. We sought to overcome this challenge by incorporating GWAS results from cell-based studies using the same chemotherapy as a clinical cohort.

Methods—In this study, we test whether the overlap between genetic variants identified in a preclinical and clinical study of capecitabine is more than expected by chance. A GWAS of capecitabine-induced cytotoxicity was performed in 164 lymphoblastoid cell lines (LCLs) derived from the CEU HapMap population and compared to a GWAS of hand-foot syndrome (HFS), the most frequent capecitabine-induced adverse drug reaction (ADR), in Spanish breast and colorectal cancer patients (n=160) treated with capecitabine.

Results—We observed an overlap of 16 single nucleotide polymorphisms (SNPs) associated with capecitabine-induced cytotoxicity ($P < 0.001$) in LCLs and HFS ($P < 0.05$) in patients, which is a greater overlap than expected by chance (genotype-phenotype permutation empirical $P = 0.015$). Ten tag SNPs, which cover the overlap loci, were genotyped in a second patient cohort (n=85) and one of them, rs9936750, associated with capecitabine-induced HFS ($P = 0.0076$).

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Conclusions—The enrichment results imply that cellular models of capecitabine-induced cytotoxicity may capture components of the underlying polygenic architecture of related toxicities in patients.

Keywords

capecitabine toxicity; genome-wide association; integrative modeling; hand-foot syndrome; lymphoblastoid cell lines

Introduction

Capecitabine, a prodrug of 5-fluorouracil, is a chemotherapeutic often used in the treatment of breast and colorectal cancers. A substantial proportion of patients suffer one of several severe adverse drug reactions (ADRs), the most prevalent being hand-foot syndrome (HFS), which occurs in nearly 30% of patients [1]. This ADR, also known as palmoplantar erythrodysesthesia or acral erythema, is characterized by tenderness, redness and swelling of the palms of the hands and soles of the feet and is the most frequent reason for dose reduction or therapy discontinuation in these patients [2-4]. Most inter-patient variability in this toxicity remains unexplained even though some of the inter-individual differences in the occurrence of HFS can be explained by patient gender, age, and local clinical practice [5-7]. Several studies have attempted to identify genetic variants associated with this frequent ADR, often using a pharmacokinetic candidate gene approach, but results have been inconclusive and/or inconsistent [8-14]. The biological mechanisms underlying HFS remain poorly understood. The identification of patients prior to beginning a capecitabine regimen that are at greatest risk for HFS would allow physicians to choose a reduced dose or an alternative treatment.

Due to their versatility, we and others have employed lymphoblastoid cell lines (LCLs) as a discovery tool in cancer pharmacogenomics [15-19]. Recently, we used enrichment analysis to demonstrate that LCLs are a useful model to study germline genetic variation that may contribute to clinical toxicity observed in patients [20]. We found that genome-wide association study (GWAS) SNPs associated with patient paclitaxel-induced neuropathy are statistically significantly enriched for SNPs associated with paclitaxel-induced cytotoxicity in HapMap LCLs [21].

In this study, we expand our LCL/patient enrichment model to another clinically important chemotherapeutic, capecitabine, by testing for enrichment of SNPs associated with the LCL phenotype of capecitabine-induced cytotoxicity in GWAS results of SNPs associated with HFS in a cohort of Spanish breast and colorectal cancer patients receiving this drug [8]. We found that SNPs associated with patient capecitabine-induced HFS are enriched for SNPs associated with capecitabine-induced cytotoxicity in HapMap LCLs. This significant enrichment confirms that LCLs are a useful model in the study of a subset of shared genes involved in this common ADR. These results are consistent with the hypothesis that the cell-based models capture components of the underlying genetic architecture for capecitabine-induced HFS. In addition, one overlap SNP, rs9936750, also associated with HFS in a

second Spanish patient cohort, and thus may be useful in prediction models of capecitabine-induced toxicity.

Materials and Methods

LCL samples and GWAS

HapMap LCLs from a population with Northern and Western European ancestry from Utah, USA (CEU1/2, n=84 and CEU3, n = 80) were treated with increasing-concentrations of 5'-deoxy-5-fluorouridine (5'-DFUR), a major metabolite of capecitabine, and cellular growth inhibition was measured by AlamarBlue (Invitrogen) as described [22]. Treatment with this metabolite is necessary because the capecitabine-activating enzyme, cytidine deaminase (CDA), is not expressed above background in LCLs. That is, the \log_2 -transformed expression signal of the *CDA* transcript cluster was <6 in greater than 90% of the 176 LCL samples measured in Zhang et al. [23]. The \log_2 -transformed area under the survival curve (AUC) was used as the capecitabine-induced cytotoxicity phenotype in the LCL GWAS. To increase genome coverage of CEU3 (HapMap r27), ungenotyped makers were imputed using the BEAGLE software, using CEU1/2 (HapMap r22) as reference [24,25]. Greater than 2 million SNPs (minor allele frequency [MAF] > 0.05 , no Mendelian errors and in Hardy-Weinberg equilibrium [$P > 0.001$]) were tested for association with capecitabine-induced cytotoxicity, using the quantitative trait disequilibrium test total association model [26].

Patient samples and GWAS

A total of 166 breast or colorectal cancer patients were recruited at the *Hospital Universitario San Carlos* in Madrid and the *Hospital Universitario Virgen de la Victoria* in Málaga, Spain [13]. All patients provided informed consent, and the study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. Capecitabine was administered according to two different schedules. Colorectal cancer patients were treated with a standard regimen (1250 mg/m² orally every 12 hours on days 1-14 every 3 weeks), while breast cancer patients were treated either with the same standard regimen or with a continuous regimen (800 mg/m² orally every 12 hours daily). HFS was graded according to the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events v3.0.

DNA samples from the GWAS cohort were genotyped using Illumina Human610-Quad BeadChips according to the manufacturer's protocols (Illumina, San Diego, USA). The GenomeStudio software package and Plink plug-in were used to extract genotype data from files created by the Illumina iScan System. Standard QC was performed as follows: all individuals with genotype call rate $<95\%$ (n=3), and all outliers in principal components analysis (n=3), were excluded [27]. This left a total of 160 individuals (117 breast cancer patients and 43 colorectal cancer patients) for analysis. SNPs with call rate $<95\%$ and MAF $< 5\%$ were excluded, as were those whose genotype frequencies departed from Hardy-Weinberg equilibrium at $P < 10^{-6}$; for SNPs that overlapped with the LCL GWAS results, the genotype intensity cluster plots were examined manually to assess reliability; genotyping quality control was also evaluated via the inclusion of duplicate DNA samples in SNP

assays, with >99% concordant observed for all SNPs. Cox regression analyses were carried out in the R statistical environment (version 2.14.0), modeling the cumulative dose of capecitabine (mg/m^2) taken up to the development of at least grade 3 HFS. Patients not experiencing HFS of any grade during capecitabine treatment were censored at the total cumulative dose received. Patients developing grade 1-2 HFS were excluded. Associations were assessed under an additive genetic model by multivariable analysis, adjusting for gender, tumor type and treatment regimen. Estimated hazard ratios (HRs) were interpreted as relative risks of developing severe HFS at a given cumulative dose.

Enrichment analysis

To test for an enrichment of cytotoxicity-associated SNPs (LCLs) among the capecitabine-induced HFS-associated SNPs (patients), we conducted a permutation resampling analysis. The patient phenotypes (cumulative capecitabine dose and HFS event) were randomly shuffled while keeping the genotype data fixed to preserve linkage disequilibrium (LD). For each of 1000 permutation replicates, the standardized Cox score statistics were recalculated for all the SNPs. For each permutation, the number of SNPs that had $P < 0.05$ in the patient data, $P < 0.001$ in the LCL data, and the same direction of effect (the same allele associated with increased HFS and increased cytotoxicity) was calculated. The overlap distribution from the simulations was compared to the observed SNP overlap to generate an empirical P-value, calculated as the proportion of permutations in which the number of LCL/patient overlap SNPs exceeds the observed number. In addition to the capecitabine LCL cytotoxicity data, we compared the patient HFS data to LCL cytotoxicity GWAS data from carboplatin, cisplatin, and paclitaxel [21,25] paper as negative controls.

Genotyping of overlap SNPs in the second patient cohort

We performed the genotyping in an independent cohort of breast and colorectal cancer patients ($n = 27$ and 58 , respectively) recruited at the *Hospital Universitario San Carlos* and the *Hospital Universitario Gregorio Marañón*, both in Madrid, Spain. All patients provided informed consent, and the study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. DNA samples were genotyped using KasPar Assays (Kbioscience) and fluorescence was determined by the sequencer Detection System 7900HT (Applied Biosystems, Foster City, USA). SNPs with call rate $< 90\%$, MAF $< 5\%$ and/or whose genotype frequencies departed from Hardy-Weinberg equilibrium at $P < 10^{-6}$ were excluded. Statistical analyses were performed as described for the first patient cohort.

Results

Enrichment of LCL cytotoxicity SNPs in patient HFS SNPs

We compared the results from a GWAS of capecitabine-induced cytotoxicity in LCLs of European ancestry [22] to those from a GWAS of capecitabine-induced HFS in Spanish breast and colorectal cancer patients. A total of 88 patients in the latter study developed grade 3 or 4 toxicity.

Neither study produced genome-wide significant results ($P < 0.05$ after Bonferroni correction) nor did the very top SNPs match between the two studies (Fig. 1). However,

through a permutation resampling analysis of the patient data that preserves linkage disequilibrium (LD), we found that the top HFS-associated SNPs ($P < 0.05$) are significantly enriched for SNPs associated with capecitabine-induced cytotoxicity in LCLs ($P < 0.001$) with consistent allelic directions of effect (Fig. 2A, empirical $P = 0.015$). The observed enrichment of 16 SNPs between the LCL and patient studies is likely capecitabine-specific, due to the HFS SNPs not being enriched for carboplatin (Fig. 2B, empirical $P = 0.608$), cisplatin (Fig. 2C, empirical $P = 0.812$) or paclitaxel (Fig. 2D, empirical $P = 0.749$) cytotoxicity SNPs. None of these three negative control drugs are known to induce HFS. Effect sizes of all 16 overlap SNPs in the LCL and patient data are reported in Table 1. Positional and eQTL information for the 16 overlap SNPs are reported in Supplementary Table S1.

Replication study in second patient cohort

We tested the 16 LCL/patient overlap SNPs for association with HFS in a second patient cohort ($n = 85$), in which 23 events of grade 3-4 toxicity were observed. The 16 SNPs were covered by 10 tagSNPs (linkage disequilibrium $r^2 > 0.8$), which were genotyped in the second patient cohort.

One of the 10 genotyped SNPs, rs9936750, was associated with HFS in the second cohort (Table 1, Figure 3). We found that the C-allele (frequency=0.27) of SNP rs9936750 was associated with severe capecitabine toxicity (HR= 4.57, 95%CI=1.49-13.93, $p = 0.0076$; Table 2). This is an intergenic SNP located 187 kb upstream of *IRX6* (iroquois homeobox protein 6) (chr16: 55358471-55364672; b37). Other genes nearby include *MMP-2* (Matrix metalloproteinase-2) (chr16: 55513081-55540586; b37) located ~341 kb upstream, *CES1* (carboxylesterase 1) (chr16: 55836763-55867075; b37) ~660 kb upstream, and the solute carrier *SLC6A2* (chr16: 55689542-55740104; b37) ~500 kb upstream. We then explored *in silico* possible functional mechanisms underlying the observed association. Upon imputation of the untyped SNPs flanking this SNP using IMPUTE version 2.0 [28], we found 12 strongly correlated SNPs ($r^2 \geq 0.8$) with similarly significant associations. We examined ENCODE data (<http://genome.ucsc.edu/ENCODE/>) in order to explore the overlapping of these SNPs with any regulatory elements (Figure S1, Supplementary Table S2). Interestingly, rs9936750 lies at a weak enhancer, while the imputed SNP rs1386257, which is in complete linkage disequilibrium (LD, $r^2=1.0$), lies at a weak promoter (Figure 4: chr16: 55165994-55189120, B37). Both SNPs are located in conserved regions and within regulatory elements specifically active in HELA G2 (hepatocellular carcinoma cell line), thus suggesting a regulatory mechanism for the associations observed. To further explore whether *CES1* could be the target gene of these putative cisQTLs, we assessed associations between them and gene expression in different human tissues. However, we observed no evidence in adipose tissue, lymphoblastoid cells, liver, brain cortex or skin, of association of rs9936750 or linked SNPs with RNA expression of *CES1*, *IRX6*, *MMP2* or *SLC6A2* (SCAN database [scandb.org], eQTL Browser [eqtl.uchicago.edu] and Genevar database, [<http://www.sanger.ac.uk/resources/software/genevar>]) [29-31].

Discussion

We performed a GWAS of capecitabine-induced cytotoxicity in LCLs and showed significant enrichment of the top cytotoxicity-associated SNPs in a clinical GWAS of capecitabine-induced HFS in breast and colorectal cancer patients. This enrichment demonstrates that susceptibilities to increased cytotoxicity in LCLs and HFS in breast and colorectal cancer patients likely have some genetic mechanisms in common and supports the role of LCLs as a preclinical model for capecitabine toxicity studies.

The SNP from the enrichment analysis that also associated with HFS in the second patient cohort, rs9936750, is in an intergenic region and is not an eQTL. The closest gene, *IRX6*, is 187 kb downstream of the SNP and belongs to the Iroquois homeobox gene (*IRX*) gene family that is involved in embryonic patterning, morphogenesis, growth, and differentiation [32-34]. However, the function of *IRX6* is currently not well characterized in humans. This gene, which is expressed in LCLs [30], has been related to Dupuytren's disease (DD), an inherited proliferative connective tissue disorder that involves the palmar fascia of the hand [35]. This thickening occurs in the palm and can extend into the fingers. Up-regulation in *IRX6* has been observed in the subcutaneous fat and in the skin overlying nodules in patients with Dupuytren's disease [35]. Further exploration of the potential relationship of this gene with HFS susceptibility may be warranted.

Another candidate gene is *CES1* located ~660 kb downstream of SNP rs9936750. *CES1* encodes the enzyme carboxylesterase 1, a serine esterase governing both metabolic deactivation and activation of numerous therapeutic agents. Further, it is responsible for the metabolic activation of a number of ester prodrugs [36]. The known role of *CES1* in capecitabine metabolism is upstream of 5'-DFUR [37], which was the compound used in the LCL cytotoxicity studies because population average expression levels of *CES1* and *CDA* are not above background levels in LCLs. Thus, the role for *CES1* in capecitabine toxicity once 5'-DFUR is formed could be limited, but we cannot rule out a role for this enzyme in downstream metabolism of capecitabine. Similarly, the previously observed association between a *CDA* promoter SNP and HFS [8] may have been missed in our downstream 5'-DFUR LCL study, although the limited sample size of both studies is also a potential cause. A major limitation of the LCL model is that most drug-induced effects involve the interaction of different cell types and organs; thus, a single system cannot represent the complexity of drug effects in the human body. However, our enrichment analysis implies that there are some common genetic mechanisms between capecitabine-induced HFS and 5'-DFUR-induced cytotoxicity in LCLs.

Matrix metalloproteinase-2 (*MMP2*) is located ~300 kb downstream of rs9936750. In tissue ulceration, *MMP-2* (72 kDa collagenase, gelatinase A) enzymatic activity is upregulated at the sites of inflammation, fibrosis, and angiogenesis [38]. *MMP2* expression has also been related to psoriasis [39] and with a rare cutaneous disease called amicrobial pustulosis of the folds (APF), which is characterized by relapsing sterile pustules frequently associated with autoimmune disorders [40]. Finally, *SLC6A* (solute carrier family 6), also known as the norepinephrine transporter (*NET*) encodes a multi-pass membrane protein, which is responsible for re-uptake of norepinephrine into presynaptic nerve terminals and is a

regulator of norepinephrine homeostasis. Several genetic studies have provided insights into how genomic variation in *NET* influences brain physiology [41,42] and pharmacology [43-45]. However, no associations with capecitabine response have been described to date.

In summary, our integrative enrichment approach that combines an LCL GWAS and clinical results from two independent cohorts of patients treated with capecitabine identified a potential regulatory SNP (rs9936750) associated with severe capecitabine toxicity. We have demonstrated that LCLs comprise a useful model system for identifying genetic variants associated with pharmacologic phenotypes, in particular severe toxicities such as HFS. The replications in independent patient cohorts of findings from these studies are essential to validate the results. This approach could be applied to find new genes influencing other drug-related phenotypes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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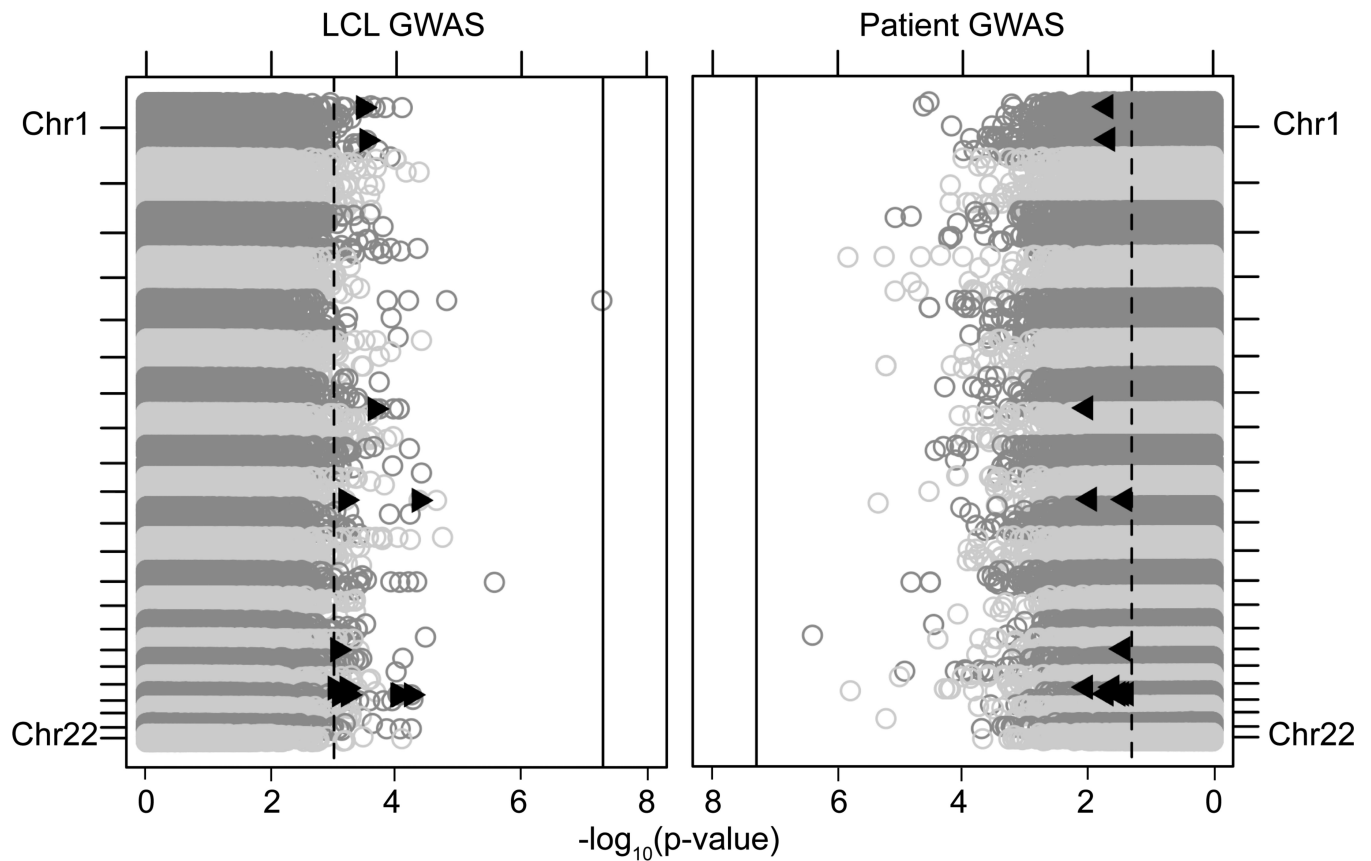


Figure 1. Comparison of individual genome-wide association study (GWAS) results

Left: capecitabine-induced cytotoxicity in LCLs. Right: capecitabine-induced HFS in patients. Black triangles correspond to SNPs with the same directions of effect in both GWAS and P-values < 0.001 in the LCL data and < 0.05 in the patient data. Dashed lines indicate the thresholds used in the enrichment analysis, while solid lines indicate genome-wide significance ($P < 5 \times 10^{-8}$).

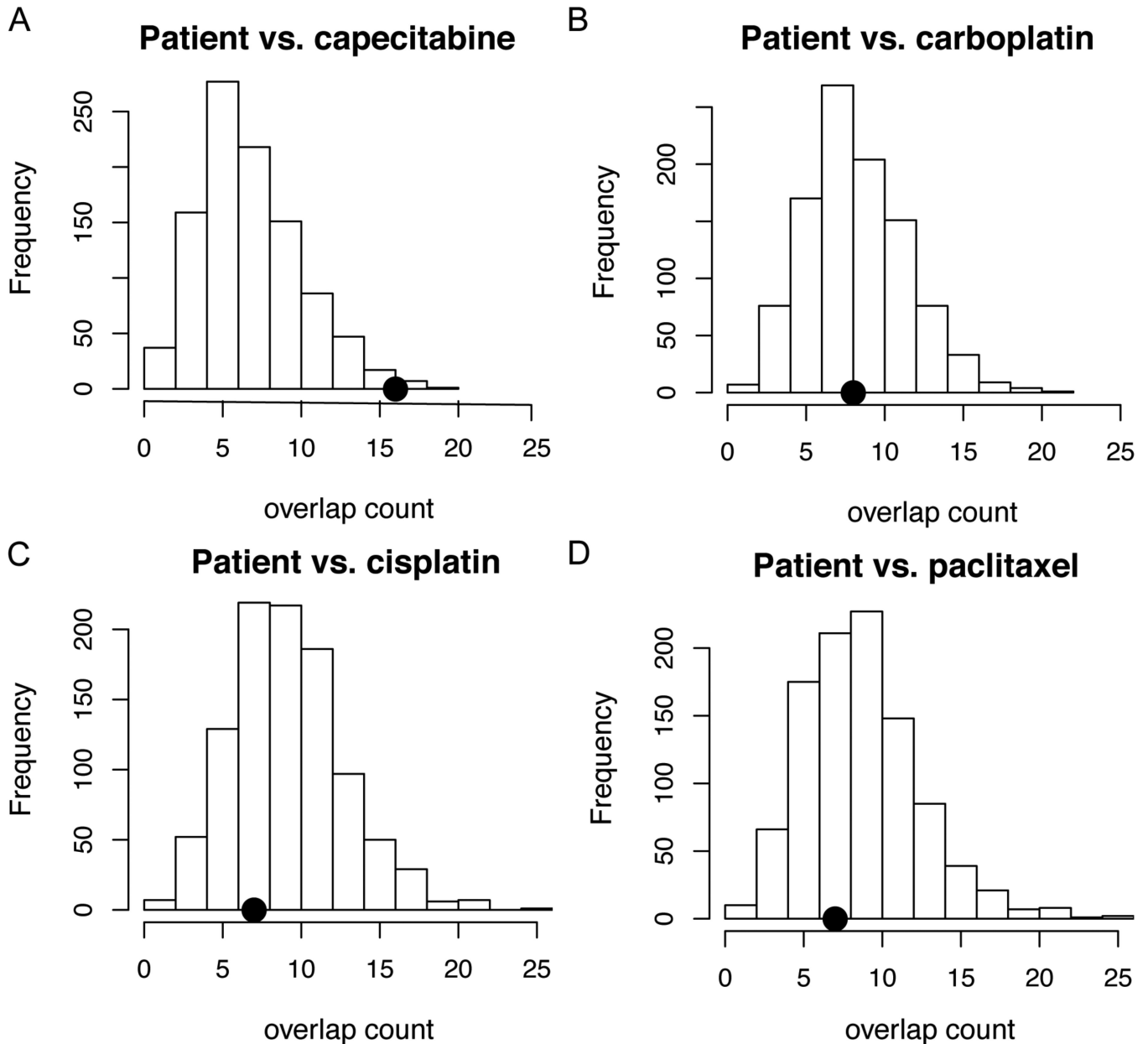


Figure 2. Patient capecitabine-induced HFS SNPs are enriched for SNPs associated with capecitabine-induced cytotoxicity in LCLs, but not for SNPs associated with cytotoxicity induced by other chemotherapeutics

Distribution of chemotherapy-induced cytotoxicity SNP count ($P < 0.001$) in 1000 permutations of the patient HFS genotype-phenotype connections ($P < 0.05$). The dot is the observed SNP overlap at these thresholds for (A) capecitabine, empirical $P = 0.015$, (B) carboplatin, empirical $P = 0.608$, (C) cisplatin, empirical $P = 0.812$, and (D) paclitaxel, empirical $P = 0.749$.

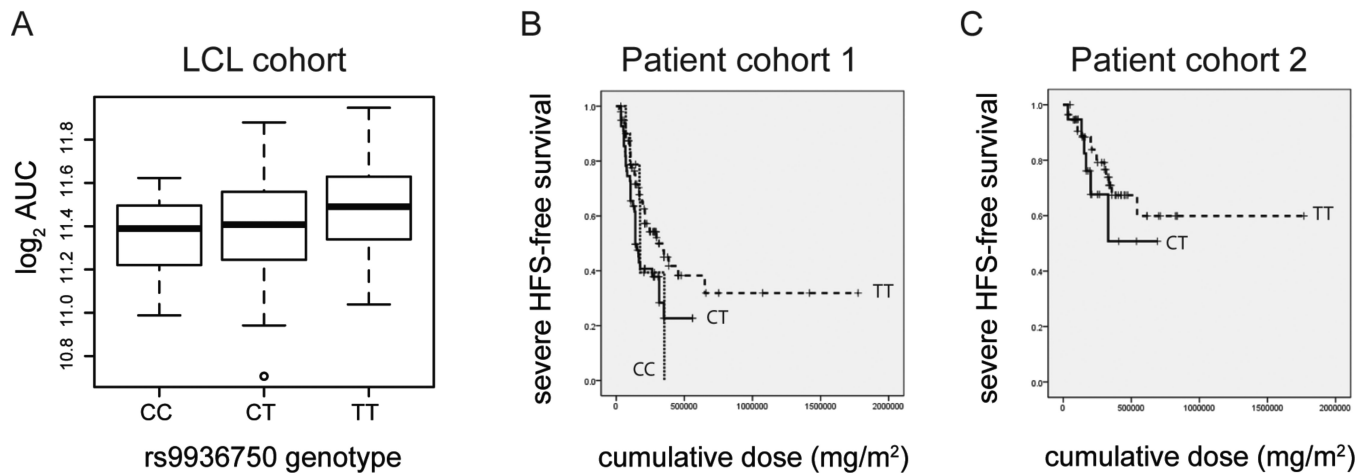


Figure 3. The association of one capecitabine patient/LCL overlap SNP, rs9936750, also associated with HFS in a second patient cohort
 rs9936750 genotype is associated with (A) capecitabine-induced cytotoxicity in LCLs ($P = 8.7 \times 10^{-4}$), as measured by area under the concentration curve (AUC). (B) Kaplan–Meier analysis of cumulative dose of capecitabine up to the development of grade 3 HFS, by rs9936750 genotype in patient cohort 1 ($P = 0.036$), and (C) in patient cohort 2 ($P = 0.0076$). The C allele is associated with increased cytotoxicity (decreased survival) and increased risk of HFS.

Table 1

Capecitabine-induced patient HFS ($P < 0.05$) and LCL cytotoxicity ($P < 0.001$) overlap SNPs and association results from a second patient HFS cohort. Tagged SNPs were not genotyped in the replication cohort.

SNP	LCL Z score	LCL P value	Patient HR	Patient P value	Replication Patient HR	Replication Patient Pvalue
rs9936750	-3.33	8.72E-04	1.39	0.036	4.57	0.0076
rs361342	-3.69	2.21E-04	1.71	0.0092	1.38	0.44
rs16864720	-3.62	2.99E-04	1.54	0.021	0.79	0.58
rs11587694	-3.58	3.39E-04	1.54	0.019	0.85	0.61
rs8083511	-3.42	6.20E-04	1.65	0.0091	1.15	0.67
rs10159569	-3.41	6.52E-04	1.54	0.011	1.1	0.74
rs8111785	-3.92	9.01E-05	1.70	0.030	1.14	0.81
rs3760779	-3.90	9.55E-05	1.68	0.035	tagged by rs8111785	tagged by rs8111785
rs166601	-.02	5.77E-05	1.70	0.030	tagged by rs8111785	tagged by rs8111785
rs10421012	-3.92	9.01E-05	1.70	0.030	tagged by rs8111785	tagged by rs8111785
rs3745660	-3.92	9.01E-05	1.65	0.041	tagged by rs8111785	tagged by rs8111785
rs10164276	-3.92	9.01E-05	1.70	0.030	tagged by rs8111785	tagged by rs8111785
rs269807	-3.35	8.21E-04	1.76	0.020	1.1	0.85
rs12052173	-3.44	5.91E-04	1.76	0.020	tagged by rs269807	tagged by rs269807
rs2864037	-4.09	4.36E-05	1.48	0.039	0.74	0.52
rs3826620	-3.30	9.65E-04	1.53	0.024	0.61	0.19