Report of Confirmation of the rs7853758 and rs885004 Haplotype in *SLC28A3*

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Aims: To validate a laboratory-developed test for the nucleoside transporter, *SLC28A3*, which has been associated with an increased risk of anthracycline-induced cardiomyopathy.

Methods: We used Taqman[®] allele discrimination to test for two variants of the *SLC28A3* gene: rs7853758 (c.1381C>T) and rs885004 (c.862-360C>T).

Results: During the validation process, we noted that several DNA samples obtained from the Coriell Cell Repository (Camden, NJ) were positive for both the c.1381 C>T and c.862-360C>T variants and another variant allele for either c.1381 C>T or c.862-360C>T (e.g., c.1381C>T homozygous/c.862-360C>T heterozygous, c.1381C>T homozygous/c.862-360C>T homozygous). We used de-identified DNA samples from trios of family members (mother, father, and child) to establish that the c.1381 C>T and c.862-360C>T variant alleles could be inherited in *cis* on the same chromosome.

Conclusions: Samples containing three variant alleles suggest that the c.1381 C>T and c.862-360C>T are in *cis* on the chromosome in some individuals and may have implications when calculating anthracycline-induced cardiomyopathy risk. In this study, we confirm a novel haplotype of *SLC28A3* using familial studies.

Keywords: *SLC28A3*, haplotype, rs7853758, rs885004, pharmacogenetics

Introduction

PHYSICIANS OFTEN OPTIMIZE THERAPY for a particular patient by a trial-and-error method and before the appropriate dose can be established some patients may experience adverse drug reactions. In the United States, more than two million cases of adverse drug reactions occur annually, accounting for 100,000 patient deaths (Lazarou *et al.*, 1998). Similarly, a large German study reported adverse drug reactions account for ~6% of patient hospitalizations (Dormann *et al.*, 2004). Patients frequently respond differently despite being treated with the same dose and this interindividual variation in drug response poses a serious challenge in health care. Currently, there are a limited number of biomarkers that can predict patient response to medications. This interindividual variability in drug response could be due to multiple factors such as disease determinants, genetic and environmental factors, variability in drug target response (pharmacodynamic response), or idiosyncratic response (Zheng *et al.*, 2007). Thus, an understanding of the variability in efficacy and toxicity of medications, especially as a result of genetic variation, may provide safer and more effective therapy.

Treatment of childhood cancer patients with anthracyclines can result in a highly variable response between indi-

viduals. In an *in vitro* study, *SLC28A3* was found to transport several anthracyclines (including doxorubicin) into cells (Nagasawa *et al.*, 2001). Genetic variants in *SLC28A3* might, thus, alter the ability of *SLC28A3* to transport anthracyclines (including in cardiac tissue). In childhood cancer survivors who received prior treatment with anthracyclines, an association was observed between two *SLC28A3* genetic variants and risk of anthracycline-related cardiomyopathy. The variant alleles for both rs7853758 (c.1381C>T) and rs885004 (c.862-360C>T) were associated with a decreased likelihood of developing anthracycline-induced cardiomyopathy (Visser *et al.*, 2012, 2013). According to Visscher *et al.* (2012), they reported a high linkage disequilibrium between rs885004 and rs7853758. After analysis of effect independence for the anthracycline-induced cardiomyopathy risk by the rs7853758 (c.1381C>T) and rs885004 (c.862-360C>T) variants, association disappeared further implicating that the variants were possibly in *cis*, but was not proven. Testing for these mutations in pediatric oncology patients before starting treatment with anthracyclines could thus help physicians to predict the risk of their patients developing anthracycline-related cardiomyopathy on an individual basis.

Owing to pharmacogenomics implications, we validated a clinical laboratory-developed assay to test for these two

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TABLE 1. ALLELIC SEQUENCES FOR TAQMAN REAGENTS

<i>dbSNP rs #</i>	<i>Sequence (5'-3')</i>
rs7853758	GACAGGGCTGAATTCATAAAAGACA [A/G]CAGGGCCAGGAAGGCAATC AGATTC
rs885004	TAGTTAGCAGAGTAACCTAAGTT GA[A/G]ATAAATTGTGAGCAAC CTCAGAGCC

variants in the *SLC28A3* gene: rs7853758 (c.1381C>T) and rs885004 (c.862-360C>T). During the validation process, we noted that several DNA samples obtained from the Coriell Cell Repository (Camden, NJ) were “triple” positives for c.1381 C>T or c.862-360C>T, suggesting that both c.1381 C>T or c.862-360C>T are in *cis* in some individuals. We used existing de-identified trio (mother, father, and child) DNA samples to confirm that the c.1381 C>T and c.862-360C>T variants could be inherited in *cis*.

Materials and Methods

Samples

A total of 189 reference DNA samples were obtained from the Coriell Cell Repositories; 52 of these samples are used as controls for Pharmacogenomics Laboratory routine patient testing and 137 samples were selected as they were part of the CDC's GeT-RM project. An additional 19 samples were obtained from Mt. Sinai School of Medicine. Seventeen existing family trios (mother, father, and offspring) were de-identified and were used for phasing studies.

DNA extraction

DNA was extracted from saliva (DNA Genotek Inc, Toronto, Canada) using the Qiagen QiAMP reagents (Valencia CA) or the Qiagen EZ1 DNA Tissue Kit, according to the manufacturer's instructions. Approximately 1 µg of DNA was extracted from saliva samples with an average concentration of about 30 ng/µL.

SLC28A3 genotyping

Two variants were chosen for the genotyping assay based on risk of developing anthracycline-induced cardiomyopathy: rs7853758 (c.1381C>T) and rs885004 (c.862-360C>T). Approximately 90 ng of DNA was amplified in singlicate by real-time PCR on the LifeTech QuantStudio 12K Flex (software v1.2.2; Grand Island, NY) and subjected to Taqman® allele discrimination using commercially available LifeTech reagents in a custom-designed open array (Table 1). All 189 Coriell reference samples were successfully genotyped

(Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/gtmb).

Sequencing

A subset ($n=11$) of genotyped samples were Sanger sequenced to validate the accuracy of the TaqMan genotyping assay. Primers for each genetic variant was designed specific to the gene of interest using the following tools: Primer 3 version 2004 developed by Rozen and Skaletsky (2000) (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), NCBI Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/), and IDT OligoAnalyzer from Integrated DNA Technologies (www.idtdna.com/analyzer/Applications/OligoAnalyzer/). Integrated DNA Technologies, Inc. (Coralville, Iowa, USA) performed synthesis of the primers. Primer sequences for each genetic variant are provided in Table 2.

PCR amplification was performed using the following conditions: initial denaturation at 98°C for 30 s, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 58°C for 10 s, primer extension at 72°C for 30 s and final extension at 72°C for 5 min. A “MyCycler™ Thermal cycler” (Bio-Rad, Hercules) was used and the PCR contained the following reagents: 10 ng genomic DNA, 1×Platinum SuperFi PCR Master Mix (Thermo Fisher Scientific, MA), and 0.112 µM of the forward and reverse primers (Integrated DNA Technologies, Inc., Coralville).

Purification of the PCR amplicons was performed using the MinElute® PCR Purification Kit (QIAGEN®, Hilden, Germany). The protocol was adjusted by performing elution twice in 20 µL of DNase-free water. Samples mixed with 0.25 µM of the primer used for sequencing were submitted to ACGT, Inc. (Wheeling, IL) for Sanger sequencing. Analysis of the sequences was performed using BioEdit biological sequence alignment editor (Ibis Therapeutics, Carlsbad).

Linkage disequilibrium analysis

The National Cancer Institute's LDlink web-based applications were used to determine linkage disequilibrium of the two *SLC28A3* variants across all populations sampled in the 1000 Genomes Project. The haplotype prevalence and the probability that the two variants are in linkage disequilibrium were both calculated. <https://analysistools.nci.nih.gov/LDlink/?tab=home> (accessed June 13, 2018)

Results

A total of 208 DNA samples were genotyped for two variants in the *SLC28A3* gene. We observed seven samples where c.1381C>T and c.862-360C>T occurred with a third variant allele (i.e., c.1381C>T homozygous/c.862-360C>T heterozygous). We noted that c.1381C>T and c.862-360C>T could also occur independently.

TABLE 2. PRIMER SEQUENCES AND PCR CONDITIONS

<i>dbSNP #</i>	<i>HGVS nomenclature</i>	<i>Forward primer sequence (5'-3')</i>	<i>Reverse primer sequence (5'-3')</i>	<i>PCR product/amplicon length (bp)</i>
rs885004	c.862-360C>T	TGTGTCTGCCATCCAGTAGG	CCTGGTGCTAAAAAGACATGG	161
rs7853758	c.1381C>T	CCCCTGACAACTCCTTGGA	CAGGGGCGTGATGTGATTAT	239

Several family DNA samples that were in the laboratory were de-identified (mother, father, and offspring) and genotyped for *SLC28A3* to resolve phasing of the c.1381C>T and c.862-360C>T variants. The mother was heterozygous for the c.1381 C>T variant and the father was homozygous for the c.1381C>T variant allele and heterozygous for the c.862-360C>T variant (c.1381C>T homozygous/c.862-360C>T heterozygous). Two of the children only inherited the c.1381 C>T variant allele. The third child was homozygous for the c.1381C>T variant allele and heterozygous for the c.862-360C>T variant (c.1381C>T homozygous/c.862-360C>T heterozygous). These results confirmed that c.1381C>T and c.862-360C>T are in *cis* within this region of high linkage disequilibrium in this family, and the haplotype containing both variant alleles is indeed present in the population. The LDlink web-based applications also confirmed that the variants are in linkage disequilibrium within the 1000 Genomes Project's population. Approximately 12.9% of samples reside in the haplotype containing both variant alleles, and the linkage disequilibrium correlation between the two variants have an R^2 value of 0.5646 and D' value of 0.9715 (Supplementary Table S2). The results of the family study's population and the 1000 Genomes' population is concordant in that both populations contain the haplotype with the two variants and both confirm linkage disequilibrium of the two variants within their populations.

Discussion

Since mutations in the *SLC28A3* gene could potentially affect its ability to transport several anthracyclines used for the treatment of childhood cancer patients, we developed a laboratory test for clinically relevant *SLC28A3* variants for pharmacogenomic testing. For well-established tests, there is consensus to which variants should be present in a clinical assay. For pharmacogenetic assays where the field is continuing to evolve, there is currently lack of consensus or standardization as to which variants should be assayed for many, if not most genes. The Clinical Pharmacogenetics Implementation Consortium (CPIC) evaluates the evidence of each variant, but does not provide a genotyping minimum standard as that is outside the purview of their charge. Currently, there are no CPIC guidelines that support *SLC28A3* testing. Thus, we chose the variants in our clinical assay based on clinical information available from association studies for each variant and the availability of commercial reagents.

We reviewed the literature for the *in vitro* study of anthracycline transport and haplotypes of *SLC28A3* were not described. In addition, www.Pharmgkb.org (accessed April 3, 2018) does not give any existing haplotypes for *SLC28A3*. Haplotypes involving the two variants we tested (c.1381C>T and c.862-360C>T) were not described in any other literature that we could find. Therefore, we propose our confirmed haplotype simply as H4, so that it can be incorporated easily when a consensus of haplotypes for *SLC28A3* is established. We designated haplotype H1 for individuals where no variant alleles were detected (i.e., wild type). H2 and H3 are haplotypes designated for each variant allele occurring independently, c.1381C>T or c.862-360C>T,

TABLE 3. PROPOSED NEW HAPLOTYPE FOR *SLC28A3*

<i>SLC28A3</i> haplotypes	<i>rs7853758</i> <i>c.1381C>T</i>	<i>rs885004</i> <i>c.862-360C>T</i>
H1	C	C
H2	T	C
H3	C	T
H4	T	T

respectively. H4 reflects the segregation of both variant alleles concurrently as seen in this phasing study.

In clinical testing, *cis* variants have been well documented as both impacting clinical phenotype and as a confounder for clinical interpretation. Some examples include *CFTR* (p.R117H and c.1210-12T[5][7][9]) (Watson *et al.*, 2004), *CYP2D6* (Yang *et al.*, 2017), and *MTHFR* (Brown *et al.*, 2005). Clinical laboratories deal with these confounders in a variety of ways. Most experimental assays involve long-range PCR techniques. As this service is not routinely reimbursed by payers, most clinical laboratories recommend in patient reports that familial studies be performed to determine phasing. Thus, we would recommend that *SLC28A3* be handled similar to *CFTR* or *MTHFR* as it is translated into clinical practice.

Visscher *et al.* use *SLC28A3* in their predictive multimarker model as a risk for anthracycline-induced cardiotoxicity. Based on our confirmation that rs7853758 (c.1381C>T) and rs885004 (c.862-360C>T) may be in *cis*, risk models that use both markers may need to be revised.

Pharmacogenomic testing incorporates molecular typing of genetic variants to help predict human variability in drug response. There are a considerable number of variants in many of the human genes associated with pharmacokinetics or pharmacodynamics of exogenous drugs. In this study, we confirm a novel haplotype in *SLC28A3* (Table 3).

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Author Disclosure Statement

The Indiana University School of Medicine Pharmacogenomics Laboratory is a fee-for-service clinical laboratory.

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