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BRIEF REPORT

Updated *DPYD* HapB3 haplotype structure and implications for pharmacogenomic testing

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Abstract

The *DPYD* gene encodes dihydropyrimidine dehydrogenase, the rate-limiting enzyme for the metabolism of fluoropyrimidines 5-fluorouracil and capecitabine. Genetic variants in *DPYD* have been associated with altered enzyme activity, therefore accurate detection and interpretation is critical to predict metabolizer status for individualized fluoropyrimidine therapy. The most commonly observed deleterious variation is the causal variant linked to the previously described HapB3 haplotype, c.1129-5923C>G (rs75017182) in intron 10, which introduces a cryptic splice site. A benign synonymous variant in exon 11, c.1236G>A (rs56038477) is also linked to HapB3 and is commonly used for testing. Previously, these single-nucleotide polymorphisms (SNPs) have been reported to be in perfect linkage disequilibrium (LD); therefore, c.1236G>A is often utilized as a proxy for the function-altering intronic variant. Clinical genotyping of *DPYD* identified a patient who had c.1236G>A, but not c.1129-5923C>G, suggesting that these two SNPs may not be in perfect LD, as previously assumed. Additional individuals with c.1236G>A, but not c.1129-5923C>G, were identified in the Children's Mercy Data Warehouse and the *All of Us* Research Program version 7 cohort substantiating incomplete SNP linkage. Consequently, testing only c.1236G>A can generate false-positive results in some cases and lead to suboptimal dosing that may negatively impact patient therapy and prospect of survival. Our data show that *DPYD* genotyping should include the functional variant c.1129-5923C>G, and not the c.1236G>A proxy, to accurately predict DPD activity.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THIS TOPIC?

Previous research indicated that the two variants linked to the HapB3 haplotype, c.1129-5923C>G and c.1236G>A, are in perfect linkage disequilibrium (LD). Thus, the benign c.1236G>A (p.Glu412=) variant was considered a reliable

surrogate tag single-nucleotide polymorphism (tagSNP) to detect the function-altering intronic variant c.1129-5923C>G.

WHAT QUESTION DID THIS STUDY ADDRESS?

The study aimed to assess LD between c.1129-5923C>G and c.1236G>A. Our results demonstrate that some patients carry c.1236G>A but not the causal variant c.1129-5923C>G. This raises the question whether testing only c.1236G>A is appropriate to accurately predict DPD activity, and whether clinical *DPYD* testing platforms should include the function altering variant c.1129-5923C>G, instead of c.1236G>A.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

The current testing strategies that only interrogate c.1236G>A may predict a false-positive decreased function DPD phenotype that could prompt inappropriate recommendations for dosing adjustments to prevent toxicity.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

DPYD testing strategies should be revised and/or updated to include the functionally relevant variant c.1129-5923C>G rather than rely on the c.1236G>A tagSNP. This will ensure DPD activity is accurately predicted while minimizing the risk of adverse events.

INTRODUCTION

Dihydropyrimidine dehydrogenase (DPD) is the initial and rate-limiting enzyme in uracil and thymidine catabolism. Because some genetic variants in the *DPYD* gene have been shown to reduce or ablate enzyme function, patients who are DPD intermediate or poor metabolizers are at an elevated risk of toxicity when treated with anticancer regimens containing fluoropyrimidines, such as intravenous 5-fluorouracil (5-FU) or its oral prodrug capecitabine. These medications have a narrow therapeutic window and can cause fatal toxicities due to compromised pyrimidine metabolism and reduced drug clearance.[1] Common adverse effects (AEs) can include leukopenia, neutropenic fever, anemia, thrombocytopenia, oral/intestinal mucositis, stomatitis, diarrhea, nausea, vomiting, hand-foot syndrome, hair loss, and dry skin. Numerous *DPYD* variants have been associated with altered DPD enzyme activity,[2] therefore individualized fluoropyrimidine therapy using pre-emptive testing is critical to identify patients at risk of developing AEs.[1–4] The Clinical Pharmacogenetics Implementation Consortium 2018 guideline update for *DPYD* genotype and fluoropyrimidine dosing recommends up to a 50% dose reduction for DPD intermediate metabolizers, that is, patients who are heterozygous for variants causing decreased function.[2,4]

The *DPYD* HapB3 haplotype is the most prevalent decreased function *DPYD* haplotype in European populations. Approximately 4% of people with European ancestry carry this allele; it is less frequent in African, East Asian, Latino, and Ashkenazim populations.[2,4,5] The HapB3 haplotype was initially described to include three intronic variants, one synonymous variant, and a deep intronic variant that impairs DPD function by disrupting pre-mRNA splicing. Due to incomplete linkage for some of the variants, PharmVar[6] more narrowly defines the HapB3 haplotype using two variants, NM_000110.4:c.1129-5923C>G (rs75017182, intron 10) that introduces a cryptic splice site and causes a splice defect, and NM_000110.4:c.1236G>A (rs56038477, p.Glu412=), a benign variant in exon 11.[1,5] A study investigating DNA samples from 3950 individuals from the Mayo Clinic Biobank demonstrated that the intronic c.1129-5923C>G variant is the causal HapB3 variant.[7] Levels of correctly spliced mRNA were reduced by 30% in carriers of c.1129-5923C>G strongly suggesting that this variant is causing alternative splicing. Furthermore, DPD enzyme function was decreased by 35% in heterozygous carriers, corroborating that this variant is the underlying cause of fluoropyrimidine toxicity.[7]

Previous literature implied that c.1129-5923C>G and c.1236G>A are in perfect linkage disequilibrium (LD),[8] which is also supported by data of the 1000 Genomes Projects per the LDpair Tool.[9] Therefore, the benign exon variant is often utilized as a surrogate tag single-nucleotide polymorphism (or tagSNP) to infer the presence of the function-altering intronic variant. Clinical genotyping of *DPYD* identified a patient with c.1236G>A but not c.1129-5923C>G. Analysis of next-generation sequencing (NGS) data from the Children's Mercy Hospital (CMH) Data Warehouse identified an additional case. Furthermore,

data from the All of Us Research Program (*All of Us*) version 7 cohort substantiated the existence of rare cases in which these SNPs are not linked and provides estimates of population frequencies.

METHODS

The clinical sample found to carry c.1129-5923C>G without c.1236G>A was submitted by St. Jude Children's Research Hospital to Right Patient Right Drug Diagnostics for pharmacogenomic testing. Genomic DNA was extracted from whole blood using the Maxwell RSC Whole Blood DNA Kit (Promega) on the Maxwell RSC Instrument (Promega) following the manufacturer's recommendations. Clinical genotyping was performed using the PharmacoScan Assay Kit and analyzed with the Axiom Analysis Suite 5.1.1.1 (Thermo Fisher Scientific) as previously described.[10] The assay interrogates 53 *DPYD* variants, including c.1129-5923C>G and c.1236G>A. *DPYD* genotype calls were made using the commercially released allele translation table (r9; Thermo Fisher Scientific; Table S1).

The patient's genotype was confirmed using whole genome sequencing (WGS). The library for WGS was constructed using germline DNA from peripheral blood and an Illumina TruSeq DNA LT PCR-Free Sample Kit and was sequenced using a paired end 2×125 bp cycle protocol and SBS technology on Illumina NovaSeq Instrument. Sequence data was aligned using BWA version 0.7.15 against hg19 human genome and 98.9% of the genome achieved greater than 30× coverage. Reads covering *DPYD* c.1129-5923C>G and c.1236G>A were manually reviewed using Integrated Genome Viewer.[11] The patient was enrolled in, and consented to, the Clinical Implementation of Pharmacogenetics study at St. Jude Children's Research Hospital (Pharmacogenetic Determinants of Treatment Response in Children with Cancer; PGEN5; NCT00730678) and the PG4KDS: Clinical Implementation of Pharmacogenetics protocol.[12,13]

The Center for Pediatric Medicine "CMH Variant Warehouse," hosted by the Children's Mercy Research Institute, contains NGS data that were generated using a variety of methods and data analysis pipelines, including, but not limited, to WGS, whole exome sequencing (WES) and targeted exome sequencing. Because the warehouse contains a variety of data sets, the database was queried for samples that are homozygous for c.1236G>A and heterozygous for c.1129-5923C>G to assure accurate detection of incomplete LD. A single sample was identified; further queries revealed the sample to be part of a trio which has been enrolled into the Genomic Answers for Kids program. All variants within the *DPYD* coding regions were retrieved for the trio from WGS using bcftools (version 1.16), GATK (version 4.3) and Variant Effect Predictor (version 109) tools for analysis. To confirm absence/presence of c.1236G>A and c.1129-5923C>G, the trio was genotyped using commercially available TaqMan assays, C_104846637_10 and C__25596099_30, as recommended (Thermo Fisher Scientific). Genomic DNA for the trio was available through the CMH Pediatric Genome Center Repository. The use of the samples for follow-up was approved by the Children's Mercy Institutional Review Board.

Short-read WGS data ($n = 245{,}394$) from the *All of Us* Researcher Workbench was utilized to identify participants who carried at least one copy of the c.1236G>A variant without a copy of the c.1129-5923C>G variant.[14,15] Genotype calls were filtered for GQ ≥ 20. Genotypes from identified participants for the two variants were subsequently confirmed orthogonally with genotype calls from the *All of Us* genotyping array data ($n = 312{,}945$). Genotype call comparisons were attempted with available PacBio long-read sequencing data ($n = 1027$) but no identified participant overlaps were found in the current release. Genetic ancestry was computed by the *All of Us* Data and Research Center using gnomAD super populations.[16,17] Variant call rate and Hardy-Weinburg equilibrium (HWE) by genetic ancestry were computed for the *All of Us* WGS and array data with cutoffs of 0.98 and 0.001, respectively, along with LD using hail version 0.2.107.[18]

RESULTS

Clinical *DPYD* genotyping revealed a case which had only one of the two HapB3-defining variants. WGS data confirmed the presence of c.1236G>A without c.1129-5923C>G in an independent blood sample from the same patient. The read depth at c.1129-5923C>G was 74× with all reads having the G allele. Read depth at c.1236G>A was 62× with 33 (53%) reads having the A allele and 29 (47%) having the reference G allele. The patient self-reported as being of mixed race of White and East Asian.

The CMH warehouse data revealed a trio of which two individuals had the c.1236G>A tagSNP but lacked c.1129-5923C>G. As shown in Figure 1, the father does not have any variants whereas the mother is homozygous for the c.1236G>A variant and heterozygous for c.1129-5923C>G; the child is heterozygous for c.1236G>A and does not have the c.1129-5923C>G variant. The mother also carries c.85T>C (p.Cys29Arg). Because the child did not inherit c.85T>C this variant can be inferred to be in *cis* with c.1129-5923C>G, which is consistent with a previous report.[19] Genotypes for c.1236G>A and c.1129-5923C>G of all three trio members were confirmed with commercially

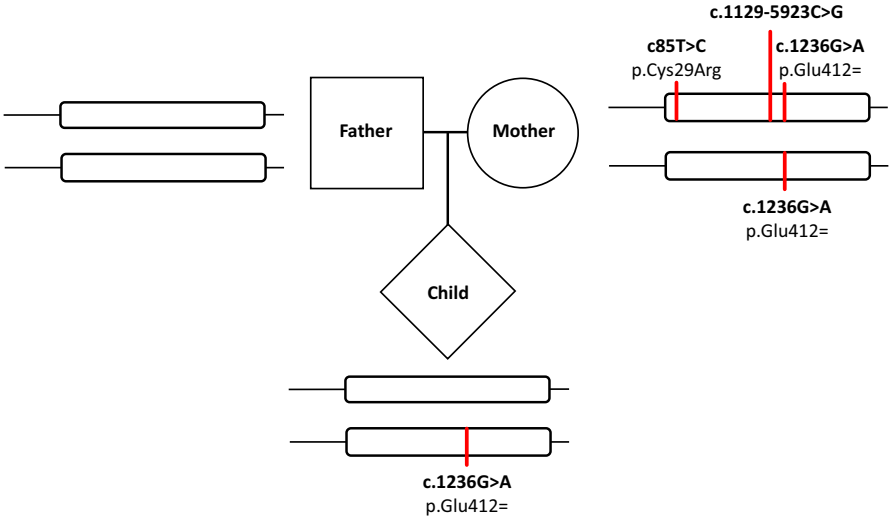

FIGURE 1 Pedigree of a trio demonstrating that c.1236G>A can occur by itself and is not in complete linkage with the causal variant, c.1129-5923C>G. As shown, the father does not have any variants while the mother is homozygous for c.1236G>A and heterozygous for c.1129-5923C>G; the child revealed only one variant, c.1236G>A, which was inherited from the mother. Inheritance also revealed that, in this case, c.85T>C (p.Cys29Arg) is in *cis* with c.1129-5923C>G and c.1236G>A. The common c.85T>C variant (gnomAD global frequency of ~28%) is classified as "normal function" by the Clinical Pharmacogenetics Implementation Consortium.

TABLE 1 *All of Us* variant frequencies computed from WGS data ($n = 245{,}394$).

Variant	Frequency overall	Afr	Amr	Eas	Eur	Mid	Oth	Sas
c.1236G>A	0.01294	0.00278	0.00555	0.00056	0.02091	0.00852	0.00920	0.01687
c.1129-5923C>G	0.01290	0.00278	0.00555	0.00056	0.02085	0.00852	0.00915	0.01687

Note: Genetic ancestry groups represent gnomAD superpopulations of African/African American (Afr), American Admixed/Latino (Amr), East Asian (Eas), European (Eur), Middle Eastern (Mid), South Asian (Sas), and Other (Oth).

available TaqMan assays. The family self-reported ethnicity as "White." Due to limited data warehouse query options, the frequency of the allele with c.1236G>A but lacking c.1129-5923C>G could not be determined.

Analysis of the *All of Us* WGS cohort corroborated our findings by identifying 14 cases with c.1236G>A whereas lacking c.1129-5923C>G (Table 1). Variant LD was 0.9985 in this data set. The 14 participants had 100% concordance with their matched array data for the two variants. Of the identified participants, 13 of 14 were of European genetic ancestry and one identified as "Other" genetic ancestry group. Among the interrogated WGS cohort, 6265 (2.55%) participants were heterozygous or homozygous for both variants. Both variants had a call rate of ≥0.99 and HWE *p* values by genetic ancestry ≥ 0.04 in both the array and WGS data.

DISCUSSION

This study unequivocally demonstrated that c.1236G>A and c.1129-5923G>A are not in perfect LD, as implied by the literature, that is, no cases have been described to the contrary. We describe several individuals possessing only one of the two variants composing the *DPYD* HapB3 haplotype. The first such case was observed through clinical genotyping using the PharmacoScan array and confirmed with WGS. Subsequently, a trio was discovered in the CMH data warehouse of which both the mother and child have c.1236G>A but lack c.1129-5923G>A. This demonstrates that the observed haplotype is heritable/germline in nature, rather than a de novo or somatic mutation.

Analysis of the *All of Us* data not only identified additional cases but also demonstrates that c.1236G>A is not a reliable marker – 0.223% of subjects with c.1236G>A lack c.1129-5923G>A – and should therefore not be utilized as a tagSNP to predict DPD function in lieu of c.1129-5923G>A. Although previous studies have been performed primarily in European cohorts,[8,19–21] not all interrogated both SNPs, which may explain why the rare allele with only c.1236G>A has just now been discovered. Additional analysis in other cohorts interrogating both variants may provide further information on population-specific LD and generate more accurate population census.

The current US Food and Drug Administration drug label for fluorouracil in the United States warns that DPD deficiency could lead to increased risk of serious or fatal adverse reactions – it is stated that fluorouracil and capecitabine should be withheld in individuals with "acute early-onset or unusual severe toxicity,"[22] and the European Union drug label states that capecitabine is contraindicated in patients with known complete absence of DPD activity. However, neither the United States nor the European Union have universal requirements or obligations for precautionary DPD deficiency testing. Additionally, neither provides specific recommendations for dose reduction in patients with a DPD intermediate metabolizer phenotype. More recently, the European Medicines Agency published a direct health professional communication in 2020 stating that DPD deficiency testing is recommended prior to 5-FU treatment. Their recommendation for genotyping includes four *DPYD* variants, c.1905+1G>A, c.1679T>G, c.2846A>T, and c.1236G>A/HapB3; these variants have been shown to cause decreased or complete absence of DPD enzymatic activity.[23] However, this statement recommends testing c.1236G>A to identify the decreased function HapB3 haplotype, and not the causal variant c.1129–5923C>G. One reason for using c.1236G>A is that the two variants have been assumed to be in perfect or near-perfect LD. Furthermore, WES only covers the exonic c.1236G>A variant, but not the deep intronic c.1129-5923G>A, and additional testing would be required to obtain results for the latter.

Given the severity of toxicity, pre-emptive testing is a powerful tool to identify DPD deficiency before treatment initiation[8,23] and, thus, future considerations should include universal recommendations for DPD deficiency testing.[3,24,25] Additionally, current guidelines need to be updated to reflect that c.1236G>A may not be a reliable variant to detect decreased DPD activity. Tests predicting DPD activity using only c.1236G>A should contain explicitly clear language stating that this tagSNP is not in complete LD with the underlying causal variant, which in rare cases may lead to a false-positive prediction of decreased DPD activity, which may trigger an inappropriate dose reduction. Reducing dose by 25% has recently been shown to lower systemic exposure and reduce efficacy.[20] Therefore, to enhance patient safety, guidelines should be updated to recommend use of c.1129-5923C>G, and not c.1236G>A for pharmacogenomic testing. The inclusion of c.1129-5923C>G in clinical testing does not impose an unsurmountable burden or challenge considering that many platforms and commercial laboratories already test both variants (for test information see the Genetic Testing Registry[26]). As platforms, instruments, and workflows, and local requirements greatly differ among laboratories, directors need to evaluate their best options on an individual basis to incorporate c.1129-5923C>G testing moving forward.

CONCLUSION

The independent discovery of multiple individuals with only one of the two HapB3 variants unequivocally demonstrates that these variants are not in perfect LD. Consequently, testing that only includes the benign variant (c.1236G>A) and not the causative variant (c.1129-5923C>G) may produce false-positive results for some patients and lead to suboptimal dosing that may negatively impact a patient's therapy and prospect of survival.

AUTHOR CONTRIBUTIONS

A.G. and A.J.T. designed the research, analyzed the data, and wrote the manuscript. E.C.B., A.H., P.E.E., and C.E.H. performed the research, analyzed the data, and wrote the manuscript. G.S., S.T., and W.Y. performed the research, analyzed the data, and U.B. and S.M.O. wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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