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Concordance between Research Sequencing and Clinical Pharmacogenetic Genotyping in the eMERGE-PGx Study

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There has been extensive debate about both the necessity of orthogonal confirmation of next-generation sequencing (NGS) results in Clinical Laboratory Improvement Amendments–approved laboratories and return of research NGS results to participants enrolled in research studies. In eMERGE-PGx, subjects underwent research NGS using PGRNseq and orthogonal targeted genotyping in clinical laboratories, which prompted a comparison of genotyping results between platforms. Concordance (percentage agreement) was reported for 4077 samples tested across nine combinations of research and clinical laboratories. Retesting was possible on a subset of 1792 samples, and local laboratory directors determined sources of genotype discrepancy. Research NGS and orthogonal clinical genotyping had an overall per sample concordance rate of 0.972 and per variant concordance rate of 0.997. Genotype discrepancies attributed to research NGS were because of sample switching (preanalytical errors), whereas the majority of genotype discrepancies (92.3%) attributed to clinical genotyping were because of allele dropout as a result of rare variants interfering with primer hybridization (analytical errors). These results highlight the analytical quality of clinically significant pharmacogenetic variants derived from NGS and reveal important areas for research and clinical laboratories to address with quality management programs. *(J Mol Diagn 2017, 19: 561–566; http://dx.doi.org/10.1016/j.jmoldx.2017.04.002)*

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Research genotyping and clinical genetic testing platforms have evolved from low-throughput targeted variation detection to full-gene sequencing, high-throughput multiplex targeted genotyping, and now to genome-wide analyses using chromosomal microarrays and next-generation sequencing (NGS). Despite the increasing availability of NGS and its capacity to interrogate multiplexed gene panels, exomes, and genomes, clinical NGS testing protocols often require identified variants to be confirmed by costly and time-consuming Sanger sequencing.[1] However, systematic analyses of NGS validation by Sanger sequencing have recently concluded that NGS is highly accurate and reliable (99.965% concordance with Sanger), and that orthogonal validation may be redundant for clinical NGS best practices.[1,2] In addition, there has been extensive debate about the return of research NGS results to study participants based on the uncertainty of variant quality (and validity), because these samples are typically not collected and/or tested by a Clinical Laboratory Improvement Amendments− and/or College of American Pathologists−approved clinical laboratory.[3]

Recent data from a small cohort that underwent either exome ($n = 176$) or genome ($n = 68$) research sequencing and pharmacogenetic genotyping in a clinical laboratory indicated that many variants included in Clinical Pharmacogenetics Implementation Consortium guidelines were highly (>95%) concordant between whole-genome sequencing and targeted genotyping before any platform optimization[4]; however, pharmacogenetic NGS panels sequenced at high depth have not been previously studied in large research cohorts with available orthogonal clinical targeted genotyping data. The eMERGE-PGx program,[5] which is an ancillary study to eMERGE phase 2,[6] was designed to detect novel variation in known pharmacogenes and to place pharmacogenetic genotyping results preemptively into the electronic health record with accompanying clinical decision support. The design included both research sequencing using the PGRNseq panel[7] and orthogonal targeted genotyping/sequencing in clinical laboratories. Consequently, the large eMERGE-PGx data set was interrogated for concordance between research NGS and clinical pharmacogenetic genotyping in an effort to inform the ongoing debates on research NGS quality and returning results derived from genomic sequencing studies.

Materials and Methods

The eMERGE-PGx Program

The design of the eMERGE-PGx program has been previously reported.[5] Institutional review board approval was obtained individually by all sites participating in the study. In brief, eMERGE-PGx had three objectives: i) deploy PGRNseq,[7] an NGS platform that provides high-quality sequence on 84 pharmacogenes, in approximately 9000 patients likely to be prescribed drugs of interest in a 1- to 3-year time frame across nine clinical sites; ii) integrate well-established, clinically-validated pharmacogenetic genotypes into the electronic health record with associated clinical decision support and assess process and clinical outcomes of implementation; and iii) develop a repository of pharmacogenetic variants of unknown significance linked to a repository of electronic health record−based clinical phenotype data for ongoing pharmacogenomics discovery. These objectives were achieved by recruiting 9015 individuals across nine clinical sites.[5] Enrollment goals at each site ranged from 250 to >1000. Because of the funding structure of eMERGE-PGx, many sites had research PGRNseq and Clinical Laboratory Improvement Amendments genotyping performed at two or more independent laboratories, providing the opportunity for comparison of concordance rates across combinations of sites and laboratories. Information about specific genetic variants typed and returned at each site has been published previously, along with information about the specific orthogonal genotyping platforms and genotyping laboratories used at each site.[5]

eMERGE-PGx Genetic Testing and Concordance

For this concordance substudy, participants from eMERGE-PGx sites were included that had at least one pharmacogenetic variant called by PGRNseq[7] in a research laboratory that was also genotyped by an orthogonal platform in a clinical laboratory. Each eMERGE-PGx site dictated which variants would be clinically genotyped and deposited into the electronic health record at its local institution. Variants in *VKORC1*, *TMPT*, *SLCO1B1*, *DPYD*, *CYP2C9*, and *CYP2C19*, all genes that are included on the PGRNseq panel were selected.[7] To maintain site privacy for this genotyping concordance substudy, specific site and laboratory combinations were deidentified. All research laboratory sites ran PGRNseq on either the Ilumina HiSeq 2000 or 2500 platform (Illumina, Inc., San Diego, CA), with a mean sequencing depth of 496×. Of note, the previously published development of the PGRNseq panel included an initial validation using 96 samples from the 1000 Genomes project, which resulted in an overall genotype concordance of 0.998.[8]

Orthogonal clinical genotyping platforms included the Illumina ADME array, custom Agena Bioscience (San Diego, CA; previously Sequenom) genotyping panels, commercial Agena Bioscience ADME genotyping panels, Sanger sequencing, TaqMan-based genotyping arrays, and custom PCR-based assays.[5] A single DNA sample had to have all reported variants concordant between research NGS and clinical genotyping for the per-sample concordance calculation, and any sample having one or more reported variants discrepant was considered discrepant for the per-sample calculation. Neither testing platform was considered a gold standard; therefore, concordance (percentage agreement) between research NGS and clinical genotyping results was measured rather than sensitivity or positive predictive value. Proportion CIs were calculated using the binomial exact calculation.

In addition, some of the eMERGE-PGx sites had resources available to not only identify genotype discrepancies, but to resolve them through repeat research NGS and/or clinical genotyping, or through Sanger resequencing as needed. Repeat testing interpretation and final determination regarding the source of genotype discrepancies were ultimately left to the discretion of the laboratory directors at each of the participating research and clinical laboratories.

Results

Research NGS and Clinical Pharmacogenetic Genotyping Concordance

Concordance between the research NGS and orthogonal clinical genotyping in eMERGE-PGx by site is presented in Table 1. Among the 4077 eMERGE-PGx subjects used in this study, the number of pharmacogenetic variants genotyped on orthogonal Clinical Laboratory Improvement Amendments platforms ranged from 6 to 27 across eMERGE-PGx sites. The overall per-sample concordance between research NGS and orthogonal clinical genotyping across the 4077 eMERGE-PGx subjects was 0.972, and the overall per-variant concordance rate across the 67,900 total pharmacogenetic variants was 0.997.

Research NGS Discrepancies

Of the 4077 eMERGE-PGx subjects included in concordance analyses, 1792 were tested at sites with available resources for repeat analyses to resolve genotype discrepancies. In this subset, all investigated discrepancies were clarified by additional follow-up and repeat testing, and subsequently attributed to either research NGS or clinical genotyping and subdivided by the three phases of molecular genetic testing: preanalytical, analytical, and postanalytical (Table 2). The preanalytical phase of testing includes all aspects of specimen handling before the initiation of genetic testing (eg, specimen collection and labeling, DNA isolation and shipment); the analytical phase includes all processes involved when performing the test (eg, test method and performance of procedures, DNA amplification and sequencing); and the postanalytical phase includes all processes involved after the testing has been completed (eg, translation of results, reporting). Among these 1792 interrogated subjects, 11 sample discrepancies were attributed to research NGS testing (6.1/1000) and all were because of preanalytical sample switches before sample shipment for processing and sequencing (Table 2), some of which were detected by the research NGS laboratories as unexpected duplicates or sex mismatches. No analytical or postanalytical variables were identified as causing sample discrepancies attributed to research NGS testing. Of the 27,158 pharmacogenetic variants reported in these 1792 subjects, the 11 preanalytical sample switches resulted in 38 total variant discrepancies attributed to research NGS testing (1.4/1000) (Table 2).

Clinical Pharmacogenetic Genotyping Discrepancies

Of the 1792 interrogated subjects tested at sites with available resources for repeat analyses to resolve genotype discrepancies, 26 sample discrepancies were attributed to clinical genotyping (15/1000), and of these, 24 (92.3%) were because of analytical discrepancies (Table 2). In addition, one clinical genotyping preanalytical sample switch and one postanalytical reporting error were identified. Notably, almost all clinical genotyping analytical discrepancies were because of rare variants underlying commercial genotyping assay primer binding sites, which interfered with hybridization and/or extension reactions and resulted in allele dropout. Most of these allele dropout cases (19 of 24) were because of one specific variant (*CYP2C19*17*; *rs12248560*) in a commercial genotyping kit used in two separate clinical laboratories. Commercial genotyping kit vendors were informed of all identified assay issues and indicated that subsequent assay versions would have redesigned oligonucleotide primers. Of the 27,158

Table 1 Summary of Research NGS and Clinical Pharmacogenetic Genotyping Concordance in eMERGE-PGx

Site	Unique DNA samples, *n*	Pharmacogenetic variants interrogated, *n*	Per-sample concordance (95% CI)*	Per-variant concordance (95% CI)
A	607	14	0.993 (0.983–0.998)	0.9995 (0.999–1)
B	442	14	0.985 (0.968–0.994)	0.9985 (0.997–0.999)
C	292	18	0.973 (0.947–0.988)	0.9937 (0.991–0.996)
D	451	16	0.960 (0.938–0.97)	0.9975 (0.996–0.999)
E	299	17	1 (0.998†)	1 (0.999†)
F	599	6	0.959 (0.939–0.973)	0.9917 (0.988–0.994)
G	226	8	0.978 (0.949–0.993)	0.9962 (0.992–0.998)
H	1052	27	0.957 (0.943–0.969)	0.9961 (0.995–0.997)
I	109	17	0.991 (0.950–1)	0.9995 (0.999–1)

Proportion CIs were calculated using the binomial exact calculation.

*Percentage of unique DNA samples concordant for all reported pharmacogenetic variants.

†One-sided 97.5% CI.

NGS, next-generation sequencing.

Table 2 Summary of Genotyping Discrepancies Attributed to Research or Clinical Genotyping in eMERGE-PGx (from Subset of Sites with Available Resources for Repeat Analyses to Resolve Genotype Discrepancies)

		Research NGS				Clinical genotyping			
Site	Total samples, *n*	Preanalytical	Analytical	Postanalytical	Sample discrepancy rate attributed to research NGS	Preanalytical	Analytical	Postanalytical	Sample discrepancy rate attributed to clinical genotyping
Sample discrepancies									
A	607	0	0	0	0	0	4*	0	0.0066
B	442	3†	0	0	0.0068	0	3‡	1§	0.0090
C	292	8¶	0	0	0.027	0	0	0	0
D	451	0	0	0	0	1‖	17**	0	0.040
Total	1792	11	0	0	0.0061	1	24	1	0.015

		Research NGS				Clinical genotyping			
Site	Total variants, *n*	Preanalytical	Analytical	Postanalytical	Variant discrepancy rate attributed to research NGS	Preanalytical	Analytical	Postanalytical	Variant discrepancy rate attributed to clinical genotyping
Variant discrepancies									
A	8498	0	0	0	0	0	4*	0	0.00047
B	6188	5†	0	0	0.00081	0	3‡	1§	0.00065
C	5256	33¶	0	0	0.0063	0	0	0	0
D	7216	0	0	0	0	1‖	17**	0	0.0025
Total	27,158	38	0	0	0.0014	1	24	1	0.00096

*In three independent DNA samples, *rs9923231* was genotyped incorrectly because of allele dropout as a result of a rare 14-bp deletion variant underlying a commercial primer binding site; a software genotype calling error; and a low-quality call despite passing overall call rate quality control (confirmed on repeat testing). In one additional DNA sample, *rs12248560* was genotyped incorrectly because of a low-quality call despite passing overall call rate quality control (confirmed on repeat testing).

†Human error during sample aliquoting before DNA being sent for NGS genotyping resulted in three independent DNA samples being switched before research NGS. As a result, one variant (*rs4244285*) was incorrect in two samples and three variants (*rs9923231*, *rs4149056*, and *rs1057910*) were incorrect in one sample. One of these DNA sample switches was detected by the research NGS laboratory as an unexpected duplicate.

‡In three independent DNA samples, *rs12248560* was genotyped incorrectly because of a nearby rare single-nucleotide variant underlying a commercial primer binding site.

§For one DNA sample, correct genotyping results were translated incorrectly in the report because of human error.

¶Human error during sample aliquoting before DNA being sent for NGS genotyping resulted in eight independent DNA samples being switched before research NGS. As a result, two variants (*rs12248560* and *rs4244285*) were incorrect in two samples, three variants were incorrect in two samples (*rs4244285*, *rs1799853*, and *rs1057910* in one case, and *rs7294*, *rs9934438*, and *rs9923231* in the other), four variants (*rs12248560*, *rs4244285*, *rs9934438*, and *rs9923231*) were incorrect in one sample, five variants (*rs776746*, *rs12248560*, *rs4244285*, *rs4149056*, and *rs7294*) were incorrect in one sample, six variants (*rs12248560*, *rs4244285*, *rs4149056*, *rs7294*, *rs9934438*, and *rs9923231*) were incorrect in one sample, and eight variants (*rs776746*, *rs12248560*, *rs4244285*, *rs1799853*, *rs1057910*, *rs7294*, *rs9934438*, and *rs9923231*) were incorrect in one sample. Two of these DNA sample switches were detected by the research NGS laboratory because of sex mismatch.

‖Human error resulted in one DNA sample being switched before clinical genotyping. As a result, one variant (*rs7294*) was incorrectly called in one sample by clinical genotyping.

**In 15 independent DNA samples, *rs12248560* was genotyped incorrectly because of allele dropout as a result of a single-nucleotide variant underlying a commercial primer binding site. In two additional independent DNA samples, *rs4244285* was genotyped incorrectly because of allele dropout as a result of a single-nucleotide variant underlying a commercial primer binding site.

NGS, next-generation sequencing.

pharmacogenetic variants reported in these 1792 subjects, the 26 sample discrepancies resulted in 26 total variant discrepancies attributed to clinical genotyping (<1/1000) (Table 2).

Discussion

The eMERGE-PGx program[5] enrolled 4077 subjects, who were subjected to both research NGS using the PGRNseq panel[7] and orthogonal clinical genetic testing, which involved testing 67,900 pharmacogenetic variants on both platforms. In addition to providing a rich resource for pharmacogenomic discovery and implementing preemptive clinical pharmacogenetic testing across the eMERGE-PGx network, this data set provided an ideal opportunity to measure concordance between research NGS and clinical pharmacogenetic genotyping, which ultimately could help inform the ongoing debates on research NGS quality and returning results derived from genomic sequencing studies. Among the pharmacogenetic variants that were interrogated by both research NGS and orthogonal clinical genotyping in

this cohort, our analysis revealed a per-sample and per-variant concordance of 0.972 and 0.997, respectively, between the two testing modalities across multiple recruitment sites and laboratories involved in eMERGE-PGx.

More important, a clear distinction between the sources of research NGS and clinical genotyping discrepancies was observed, as preanalytical errors were the cause of all research NGS discrepancies and analytical errors were the cause of the majority of clinical genotyping discrepancies. The interrogated variants from the PGRNseq panel [which were sequenced to a high average depth (496×)] had excellent analytical performance,[7] and all discrepancies attributed to research NGS were the result of preanalytical sample switches. Typically, research NGS DNA samples were shipped in 96-well plates to the NGS laboratories; some of the research laboratories involved in sample acquisition, DNA extraction, and sample aliquoting did not have the same level of preanalytical quality assurance plans involving sample accessioning, labeling, handling, and storage that would be standard practice in clinical laboratories.[9–11] The absence of these practices likely contributed to the human errors and downstream genotype discrepancies attributed to the research NGS testing.

Preanalytical (and postanalytical) errors were minimal among the discrepancies attributed to clinical genotyping, whereas the majority of errors were determined to be analytical in origin. The most common analytical variable identified by follow-up testing, local sequence interrogation, and assay inspection was the presence of a low-frequency germline variant underlying an oligonucleotide hybridization or extension primer binding site. Most clinical pharmacogenetic tests for eMERGE-PGx used either commercial or custom hybridization/amplification-based genotyping assays, and the presence of a variant underlying a primer site is a known cause of allele dropout in molecular diagnostics.[12–15] A neighboring single-nucleotide variant underlying a primer site often results in preferential amplification of the other allele, which can lead to the unfortunate outcome of unknowingly detecting only a single allele and, therefore, reporting an incorrect genotype at that location. Careful primer design to avoid known variant nucleotides is standard practice in clinical laboratories; however, most commercial companies often do not disclose the primer sequences of their assays, as was the case for one of the clinical pharmacogenetic variants that experienced allele dropout by two different clinical laboratories in eMERGE-PGx (*CYP2C19*17*; *rs12248560*). When we removed discrepancies at this one variant for a sensitivity analysis, this increased the per-sample concordance in the subset of 1792 participants available for repeat testing from 0.979 to 0.990.

Furthermore, even when all known regional variant nucleotides are avoided, an amplification-based molecular assay will never achieve perfect universal specificity and sensitivity because of the presence of rare variants and subpopulation/ethnic-specific variants that have yet to be cataloged in current human genome variation databases [eg, the Short Genetic Variation Database (dbSNP) and Exome Aggregation Consortium (ExAC)]. Our concordance data highlight the importance of careful primer design, thorough assay validation, and continued surveillance of databases for variants within the oligonucleotide sequences of primers used in clinical targeted genotyping assays. They also highlight the importance of continued public sharing of novel genetic variants through databases like ClinVar.[16]

In conclusion, our concordance analysis of 4077 subjects and 67,900 reported pharmacogenetic variants generated from research NGS and clinical genotyping highlight the accuracy of the eMERGE-PGx program database and support future discoveries made from the 84-gene PGRNseq data within this resource. Moreover, these data indicate that many important pharmacogenetic variants are amenable to high-quality interrogation by short-read NGS on the PGRNseq panel (eg, *CYP2C9*, *VKORC1*, *CYP2C19*, *SLCO1B1*); however, genes known to be challenging to interrogate were not included in this analysis (ie, *CYP2D6*, *HLA*). The excellent analytical quality of the research NGS data also support recent studies that suggest routinely used confirmatory orthogonal validation is likely not necessary for genes amendable to NGS genotyping within a clinical laboratory setting where preanalytical errors are minimized.[2] In addition, the discrepancies that were resolved by our concordance substudy revealed the most important areas to address and monitor with quality management programs by both research and clinical laboratories, particularly as clinical research sequencing and precision medicine studies that include returning results to participants are increasingly being deployed by both national and international genomics programs.

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