New cytochrome P450-2D6*56 allele identified by genotype/phenotype analysis of cryopreserved human hepatocytes

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Running Title: Identifying CYP alleles with functional relevance

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Abstract

Genotype/phenotype analysis with human hepatocytes has identified a new inactive \textit{CYP2D6} allele, \textit{CYP2D6*56}. Cryopreserved human hepatocytes from 51 livers were evaluated for \textit{CYP2D6} activity with dextromethorphan as the probe substrate. Hepatocyte lots that lacked \textit{CYP2D6} activity were further evaluated for \textit{CYP2D6} expression and known genetic variations, including \textit{CYP2D6*2,*3,*4,*5,*6,*7,*8,*9,*10,*11,*14,*15,*17,*18,*19,*20,*25,*26,*29,*30,*35,*40,*41,*43} and various multiple copy \textit{CYP2D6} alleles \textit{(*1xn, *2xn and *4xn)} by the AmpliChip CYP450 prototype microarray. Two discrepancies were uncovered between the \textit{CYP2D6} genotype and activity by this approach. In one sample, a previously unreported 3201C→T transition in exon 7 resulted in Arg344(CGA) being replaced by a stop codon (TGA), resulting in a \textit{CYP2D6} enzyme lacking the terminal 153 amino acids. This allele was given the designation of \textit{CYP2D6*56} and the GenBank accession number DQ282162. The lack of \textit{CYP2D6} activity in cryopreserved hepatocytes and microsomes found in the second sample, despite a normal level of \textit{CYP2D6} expression and a genotype \textit{(*10/*1)} predictive of normal \textit{CYP2D6} activity, was attributed to enzyme inactivation by an unknown metabolite. The identification and characterization of the \textit{CYP2D6*56} allele indicates commercial cryopreserved human hepatocytes may provide a valuable means to rapidly identify genetic variations with functional relevance. This integrated approach of identifying alleles and examining allele relationships to gene expression and function could be of tremendous value to understanding the mechanism responsible for functional differences in gene variation. The commercial availability of human cryopreserved hepatocytes also makes this potential readily available to any who are interested in it, not just those with access to private liver banks.
Introduction

There have been several methods by which new gene variations have typically been discovered in human drug metabolism enzymes. DNA repositories have been sequenced for variations in a gene and from these sequences new variations in genes have been identified (Chevalier D 2001; Kiyotani K 2002; Solus JF 2004; Soyama A 2005). While effective in identifying new gene variations, many gene sequences must be evaluated that have no functional variations and some important variations are likely missed because the entire gene and regulatory region is seldom sequenced, particularly for very large genes. Furthermore, unless the new gene variations that are discovered produce an obvious effect on gene expression, the importance of the new gene variation is often unclear. Even the linkage between one gene variation and another variation in a different part of a gene (allele haplotype) is often unknown (Fernandes-Salguero P 1995; Marez D 1997; Chevalier D 2001; Solus JF 2004). A second approach has identified new alleles by evaluating a specific gene in individuals who exhibit an obvious functional difference in a gene product (de Morais SMF 1994; Oscarson M 1997; Ferguson RJ 1998; Ibeanu GC 1999; Dickmann LJ 2001; Kidd RS 2001; Pitarque M 2001; Oscarson M 2002; Gaedigk A 2003; Gaedigk A 2005). This is a far more directed approach. The focus resides with evaluating only genes from individuals who have already been defined as very different from the rest of the population. A specific phenotypic trait is directly associated with a specific allele. However, the discovery of these alleles typically requires administering a drug or other substance to humans and assessing how a specific phenotype affects its disposition. The phenotype depends on the use of probes that are safe to administer to humans and that are substantially affected by the allele that produces the altered function. The discovery of these alleles seldom allows one to address the mechanism by which a functional change is produced (altered gene product, level of gene
expression, mRNA level etc.). Those issues must often be addressed by in vitro methods (Sullivan-Klose TH 1996; Ariyoshi N 2001; Pitarque M 2001; Ramamoorthy Y 2001) that may or may not provide a totally satisfactory answer about the mechanism. Fewer efforts have been directed toward using human liver tissue to identify dysfunctional gene variations (Marez D 1997; Hustert E 2001; Haberl M 2005) and when such tissue has been used it has come from liver banks not generally accessible to the average investigator. Only recently has the entire process of using stored human liver tissue to evaluate gene expression, function, mRNA and gene sequence been truly integrated to understand genetic variation in a CYP gene (Haberl M 2005). Although other investigators have suggested human hepatocytes might be useful for examining CYP2D6 genetic polymorphisms (Komura H 2005), their actual application to identify new CYP2D6 alleles and the molecular basis for their functional effects has yet to be realized or generally appreciated for their broader application to functional genomics research.

Since the completion of the human genome project, the next great challenge rests with the efficient discovery and characterization of alleles in the human genome that are dysfunctional. This discovery and characterization would be facilitated if it was possible to evaluate genes and the mechanism(s) responsible for how or if a new allele elicited a functional change in the gene product. The current studies were undertaken to determine if commercially available cryopreserved human hepatocytes could be used in genomic research of metabolic enzymes. Specifically, whether enzyme activity from human cryopreserved hepatic tissues correlates with genotype information from that same tissue: if it is possible to relate enzyme level with activity in cryopreserved human hepatocytes; and if detailed in vitro information about a gene, its expression and its function could provide a viable approach for more efficient discovery of new gene variations of functional relevance in human metabolic enzymes. Our results indicate that
this approach provides a promising means to approach the search for new alleles of a gene expressed in the liver, based on differences in \textit{in vitro} protein activity and expression as the driver for locating new alleles with a dysfunctional effect.

\section*{METHODS}

\textbf{Human hepatocytes:} Investigational Review Board (IRB) approval was obtained from the University of Kentucky for exemption of commercially available cryopreserved human hepatocytes from the need to obtain a consent form prior to their purchase and testing for gene variations in metabolic enzymes. In Vitro Technologies, Inc. (Baltimore, MD) supplied 51 different cryopreserved human hepatocyte lots previously evaluated for CYP2D6 and CYP2A6 enzyme activities with the standard probes (dextromethorphan O-demethylase and coumarin 7-hydroxylation, respectively). Hepatocyte enzyme activities for each hepatocyte lot are available from the IVT web site at (http://www.invitrotech.com/characterizationtab.cfm#2). IVT has undertaken an extensive evaluation of their cryopreserved human hepatocytes to confirm enzyme activities are reproducible and that fresh and cryopreserved human hepatocytes (n=30) are strongly correlated (slope of approximately 1.0) (Pham C 2000). This has eliminated the need to perform these assessments by individual labs.

\textbf{Chemical supplies:} Midazolam, propranolol and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO). Bufuralol was obtained from Ultrafine Chemicals (Isle of Palms, SC). Deuterium labeled 1’OH midazolam was synthesized by Eli Lilly Company.

\textbf{Human microsomes:} Cryopreserved human hepatocytes (5 x 10^6 cells) were thawed in 37°C water bath and centrifuged at 500g for 5min. The resulting pellets were re-suspended in 1ml ice-cold Buffer A (100mM Tris-HCl; pH 7.5, 0.5mM EDTA) and homogenized with homogenizer.
The cell debris was removed at 35,000g (30min) and microsomes prepared from the 105,000g fraction before suspending in 100µl Buffer A. Protein concentration was measured by a Coomassie protein assay and the microsomes frozen. Microsomes from one hepatocyte lot (PFM) were further evaluated for CYP2D6 and CYP3A4 activity.

**Bufurolol and Midazolam assays:** Human microsomal formation of 1’OH bufurolol and 1’OH midazolam from the PFM hepatocyte lot was compared with these same activities in three separate microsome preparations from different human livers purchased from Xenotech (Kansas City, KS). Enzyme activities were compared at V_{max} concentrations for bufurolol (100 µM) and midazolam (50 µM) at 0.1 or 0.05 mg protein/mL, respectively. Microsomes were preincubated at 37°C for 3 minutes prior to initiation of the reaction with 1 mM NADPH. After a 30 min bufurolol incubation and a 1 min midazolam incubation (linear conditions for both metabolites with protein and time) the reaction was stopped with 1 mL of methanol. Samples were mixed and centrifuged and a 30 µL volume mixed with either 170 µL of deuterated 1’OH midazolam in 25% methanol (1’OH midazolam assay) or propranolol (1’OH bufurolol assay) in water. 1’OH bufurolol and 1’OH midazolam were assayed by LC/MS Sciex API. 1’OH bufurolol was assayed using an ODS column and a gradient mobile phase containing mobile phase A (5 mM ammonium acetate in 95% water/5% methanol) and mobile phase B (5 mM ammonium acetate in 95% methanol/5% water). The two mobile phases were mixed in proportions of 90%A/10%B (0-0.25 min hold), and then a gradient from 10%B to 80%B from 0.25 to 1.5 min at a flow rate of 0.22 mL/min. Mass spectrum monitoring was carried out for 1’OH bufurolol at m/z 278/186 and propranolol at m/z 260/116.2. 1’OH midazolam was assayed with an ODS column and a gradient mobile phase consisting of mobile phase A (50 mM ammonium acetate in 95% water/5% methanol) and mobile phase B (50 mM ammonium acetate in 95% methanol/5% water).
DMD#9548
water). The two mobile phases were mixed in proportions of 35%A/65%B (0-0.4 min hold, and
then a gradient from 65%B to 95%B from 0.4 to 3.0 min at a flow rate of 0.22 mL/min. Mass
spectrum monitoring was carried out for 1’OH midazolam at m/z 342/324 and for its internal
standard at m/z 347/329.

**DNA isolation and genetic testing:** Human cryopreserved hepatocyte lots were rapidly thawed
at 37°C and immediately diluted into 49 mL of thawing buffer (InVitroGRO HT medium, In
Vitro Technologies, Inc., Baltimore, MD). The thawed hepatocytes were centrifuged at 2-
3,000xg and the thawing buffer removed. The hepatocyte cellular pellet was immediately
processed and DNA isolated from the thawed hepatocytes with a Qiagen DNA mini kit for tissue
dNA extraction according to the manufacturer’s instructions. Typically, 150-200 µL was
recovered from a single vial of human hepatocytes (5,000-15,000 DNA copies per µL). The
isolated genomic DNA was initially characterized for *CYP2D6* alleles *1xn*, *2xn*, *4xn*, *2; *3,
R 1996; Chou WH 2003; Cai WM 2006) and the AmpliChip P450 prototype microarray. For
comparative purposes *CYP2A6* *2,*3,*4,*5, *9 and *12 alleles were also assessed in these
samples (Fernandes-Salguero P 1995; Rao Y 2000; Paschke T 2001; Goods S 2002; Oscarson M
2002). Two samples (WWM and PFM) were further evaluated by the P450 AmpliChip
prototype microarray for an extended group of additional *CYP2D6* alleles
(*8;*11;*14,*15,*18,*19,*20, *25,*26,*29) when CYP2D6 activity and genotype did not match.

**Immunoassays:** Additional hepatocytes were obtained from In Vitro Technologies, Inc. for
purposes of qualitatively assessing cytochrome P450 enzyme expression. Supersomes
expressing either cytochrome P450-2D6 or CYP2A6 enzyme were used as a reference and
immunoassay kits for each enzyme (Western immunoblotting kits) were purchased from BD-
Bioscience (San Jose, CA). Hepatocytes were used to make a microsomal pellet as described before and assayed by the Coomassie method for protein amounts (Loffler BM 1989). Twenty-five µg of microsomal protein was fractionated by electrophoresis on a 12% SDS-PAGE gel for 2 hrs at 120 V. Proteins were electroblotted to nitrocellulose, the nitrocellulose was blocked with 2% powdered nonfat milk and probed by immuno-specific antibodies for cytochrome P450-2D6 or CYP2A6 according to the manufacturer’s instructions.

**Cloning, isolation and sequencing:** A 6 kb fragment containing the complete CYP2D6 gene was amplified from genomic DNA by long range PCR with the GeneAmp XL PCR kit (Applied Biosystems, Branchburg NJ) and the primers CYP2D6F (5′- AGC TTT GTC GAC GAA TTC AAG ACC AGC CTG GAC AAC TTG G) and CYP2D6R (5′- AAA ACG CGG CCG CTC AGC CTC AAC GTA CCC CTG TCT CAA ATG). The reactions consisted of 100 ng genomic DNA template, 200 nM of each primer, and reaction buffer containing 0.9mM Mg(oAC)2, 200uM each dNTP, and 2U rTth DNA Polymerase, XL. The cycling parameters for these reactions consisted of 1 cycle at 94°C for 1min, followed by 16 cycles of 94°C for 0.5 minutes and 68°C for 6.5 minutes, followed by 14 cycles of 94°C for 0.5 minutes and 68°C for 6.5 minutes, with 15 seconds added to each subsequent 68°C extension step. Amplified fragments were resolved on preparative 1% agarose gels, and the 6.0 kb CYP2D6 amplicon was excised and purified by use of the Qiaex II protocol (Qiagen, Chatsworth CA) according to manufacturer’s instructions. The purified amplicon was subsequently cloned into the pCR4BluntTOPO vector (Invitrogen, Carlsbad CA) and transformed into chemically competent TOP10 cells (Invitrogen, Carlsbad CA).

Clones were screened by evaluating each CYP2D6 clone at position 100 in exon 1 by PCR-RFLP analysis. PCRs were performed with 20 ng of plasmid DNA template and the primers...
CYP2D610F (5'- GTG TGT CCA GAG GAG CCC AT) and CYP2D610R (5'- TCT CAG CCT GGC TTC TGG TC), resulting in the amplification of a 310 bp fragment. The reactions included 1.8 mM MgCl₂, 200µM each dNTPs, and 0.75U AmpliTag Gold (Applied Biosystems, Branchburg NJ). The cycling parameters for these reactions consisted of 1 cycle at 95°C for 5min, 30 cycles of 95°C for 15sec, 60°C for 30sec, and 72°C for 30sec, and a final cycle at 72°C for 10min. Aliquots of the PCR products were digested with HphI (5U, New England Biolabs Beverly, MA), and the digestions were analyzed on a 2% Metaphor gel (Cambrex Bioscience Rockland ME). Clones containing the wild type C at position 100 (*2 allele; WWM or *1 allele PFM) led to cleavage of the 310 bp fragment into sub-fragments of 230 and 80 bp, while clones with a T at position 100 (*4 allele; WWM; or *10 allele; PFM) were not cleaved.

DNA sequence analysis of 100C clones (*2 allele, WWM; and *1 allele, PFM) were performed by cycle sequencing with BigDye v3.0 chemistry and custom oligonucleotide primers. Reactions were analyzed on an ABI Prism 3700 DNA Analyzer (Applied Biosystems, Branchburg NJ), and data was assembled with the Sequencer analysis software. Sequencing was carried out in the forward and reverse directions and each SNP was re-evaluated to confirm the accuracy of the final sequence.

RESULTS

The distribution of hepatocyte CYP2D6 activity exhibited a bimodal distribution, with 12% of the hepatocyte lots exhibiting virtually no CYP2D6 activity (<2 pmol/min/10⁶ cells; Log (CYP2D6 activity) <0.3) while the remaining 88% of hepatocytes expressed a dextromethorphan O-demethylase activity that ranged from 5 to 47 pM/min/10⁶ cells (18 ± 9 pmol/min/10⁶ cells; Log (CYP2D6 activity = 1.3); Fig. 1). The majority of the hepatocyte lots (4/6; 67%) without
significant CYP2D6 activity were identified by genetic tests to contain 2 non-functional CYP2D6 alleles (*4/*4 and *3/*4, cross-hatched bars). However, an extensive CYP2D6 genotype analysis could not explain why the remaining two lots from two Caucasian samples (PFM (*10/*1, stippled black bar); and WWM (*4/*2, gray bar) had no CYP2D6 activity. Immunoblots indicated the PFM (*10/*1) hepatocyte lot expressed significant quantities of CYP2D6 enzyme, but efforts to measure CYP2D6 activity showed a consistent lack of dextromethorphan metabolism in these hepatocytes. Microsomal measurements from this lot showed significant CYP3A4/A5 activity (1’OH midazolam, 591 pM/min/mg protein), but no measurable 1’OH bufurolol formation, a specific indicator of CYP2D6 activity (<5.8 pmol/min/mg protein, limit of assay quantification). No CYP2D6 activity was detected in hepatocyte lot WWM (*4/*2) (Fig. 2A) although significant quantities and activity of CYP2A6 were observed in this lot and the PFM lot (Fig. 2B). The initial classification of the WWM hepatocyte lot as expressing a CYP2D6*2 allele was based on the SNP pattern in this allele being typical of a CYP2D6*2 allele (-1584G;-1235G;-740T;-678A; CYP2D7 gene conversion in intron 1;1661C; 2850T; 3384A; 3584A; 3790T; 4180C). However, allele cloning and sequencing on separate occasions confirmed an additional point mutation at position 3201 in exon 7 that changed 3201CÆT and the CGA(Arg344)ÆTGA(Stop) codon (Fig. 3), explaining the lack of CYP2D6 enzyme expression in figure 2A for this hepatocyte lot. The PFM *1 allele had the typical profile expected for a *1 allele (-1235A; 310G; 746C; 843T, 1661G, 3384A, 4180G) and no other variations in any exons or at intron-exon borders that would account for the complete lack of CYP2D6 enzyme activity in this lot. The only questionable variation was a G deletion in about the middle of intron 7, a variation unlikely to affect CYP2D6 expression or activity since it fell in the run GGGTGGGGGTG in intron 7.
The majority of hepatocyte lots exhibited significant overlap between the CYP2D6 genotype and predicted CYP2D6 activity (*3/*1; *4/*1; *3/*2; *4/*2; *10/*10; *41/*10, gray bars; moderate activity; and *1/*1; *1/*2; *1/*35; *2xn/*1; *2/*2. stippled black bars; normal to high activity). However, moderate CYP2D6 activity genotypes expressed a lower average CYP2D6 activity (gray bars, Mean = 13±7 pmol/min/10^6 cells; Log (CYP2D6 activity) = 1.1) relative to the hepatocyte lots with genotypes predicting a higher rate of CYP2D6 activity (stippled black bars, Mean = 22±10 pmol/min/10^6 cells; Log (CYP2D6 activity) =1.3; p<0.01 by Wilcoxon rank sum test). The association between genotype predicted and quantified CYP2D6 activity makes outliers in a group of hepatocyte lots (like the PFM and WWM) immediately apparent (Fig. 1, samples marked with an asterisk). A similar bimodal distribution was seen for other CYP enzyme activities (CYP1A2, CYP2A6, CYP2C9 -- data is not presented but it is easily obtained from IVT website which lists enzyme activities for various CYP enzymes in their banks).

The distribution of CYP2A6 activity also exhibited a bimodal shape, with 12% of the hepatocyte lots exhibiting little CYP2A6 activity (<5 pmol/min/10^6 cells; Log (CYP2A6 activity <0.7) and the remaining 88% of hepatocyte lots expressing a coumarin 7-hydroxylase activity ranging from 9 to 135 pmol/min/10^6 cells (53 ± 32 pmol/min/10^6 cells; Log (CYP2A6 activity) = 1.7). The lots exhibiting low CYP2A6 activity were not the same lots that expressed low CYP2D6 activity. Only 2/6 (33%) of the low CYP2A6 activity hepatocyte lots could be explained based on the CYP2A6 alleles tested (*1/*5 and *9/*9). This suggests additional undefined CYP2A6 alleles may be present in the remaining 4 hepatocyte lots with low CYP2A6 activity. In the case of two lots (EVY and RML), the low CYP2A6 activity was also associated with virtually no detectable CYP2A6 expression (Fig.2B). This indicates the default allele designation of CYP2A6*I is incorrect. The CYP2A6 evaluations were done to provide evidence...
that differences in hepatocyte lot activity did not represent a general depression of all CYP enzyme function, and to show that this approach need not be limited to just CYP2D6 activity and genotype assessments (a single gene product).

DISCUSSION

The identification of a new *CYP2D6* allele and its functional relevance was greatly facilitated by the ability to screen hepatocytes for enzyme activity, enzyme expression and the known dysfunctional alleles in each lot. The new *CYP2D6* allele (in lot WWM) is expected to be a rare allele, since it has not been previously reported in GenBank, the CYP450 website or the SNP database and in spite of several large efforts aimed at sequencing CYP2D6 exons, this specific variation has never previously been observed. All known inactive CYP2D6 alleles related to the CYP2D6*2A* allele reported to date (*11,*12,*19, *20,*21) are rare (<0.1% in the population) and race specific. However, it will be desirable to use banked human DNA to establish the frequency of newly identified dysfunctional alleles after these are identified by such in vitro methodologies. We opted not to do that in this instance because of the anticipated low frequency for the occurrence of this new allele.

The inability to detect CYP2D6 activity in hepatocyte lot PFM, which contained a normal CYP2D6 allele, is more difficult to explain. It is hypothesized the lack of activity in this lot resulted from selective inactivation of the CYP2D6 enzyme. This hepatocyte lot came from a 57 year old Caucasian female patient with multiple medical problems (heart valve replacement, hysterectomy, type II diabetes and hypertension). Her medical information also indicates she did not smoke, drink or use illicit drugs. The likelihood is this patient was being treated with a variety of drugs for her multiple medical problems, and one of these medications was
biotransformed into a selective and reactive metabolite that irreversibly bound to the CYP2D6 enzyme and inactivated it. This would account for the relatively normal levels of CYP2D6 enzyme or even slightly elevated levels, but a CYP2D6 activity that was absent based on separate hepatocyte (n=2) and microsomal (n=3) assessments. Reports of normal or even elevated levels of CYP3A enzyme after treatment of humans with drugs that irreversibly inactivate the CYP3A enzyme was reported decades ago (Larrey D 1983; Watkins PB 1985). This would also explain why other CYP enzymes (CYP2A6 and CYP3A4) had fairly normal activity. More detailed assessments of the CYP2D6 enzyme from this hepatocyte lot to uncover the specific mechanism for this isozyme specific inactivation was beyond the scope of this research. It was also unlikely mRNA levels would have been useful in understanding the low CYP2D6 activity in sample PFM, since alternative splicing or diminished CYP2D6 mRNA would have lowered and not resulted in normal or elevated CYP2D6 protein amounts in this hepatocyte lot. Quantification of mRNA will have the most value when the mechanism responsible for poor gene expression is unclear.

The identification of a rare new CYP2D6 allele illustrates the value of this approach to functional genomics. The ability to compare and relate enzyme activities from cryopreserved human hepatocytes with hepatocyte genotype and level of enzyme expression provides a simple means to quickly identify samples with gene variations producing dysfunctional effects. In 100% of hepatocyte lots without measurable CYP2D6 enzyme expression or activity, the effect could be predicted by alleles preventing enzyme expression. In one hepatocyte lot (PFM) where enzyme expression and activity were not related, the absence of CYP2D6 enzyme activity could be hypothesized to result from concurrent prescribing of multiple medications. In general, hepatocyte lots with diminished CYP2D6 enzyme activity had genotypes that were predictive of
less enzyme expression, while hepatocyte lots with the most CYP2D6 enzyme activity had genotypes indicative of normal or elevated enzyme levels. When searching for the proverbial “needle in a haystack” (i.e. rare or unrecognized alleles with functional relevance) the search is facilitated tremendously by employing an approach that allows one to readily identify phenotypic enzyme variation while simultaneously addressing the mechanism responsible for that variation. Therein are the two major advantages of this in vitro method over many other approaches. It is the greater information obtained and the broader application of the method that embodies the main attributes of this particular methodological approach. The focus and relevance does not reside with “what was found” but rather “how it was found”.

It was decided not to quantify mRNA in this study to address the mechanism for either of the two hepatocyte lots without CYP2D6 activity (WWM and PFM). However, quantification of a specific mRNA could be done to help further elucidate mechanisms responsible for differences in allele expression when interpretation of the precise genetic change or basis for the differences in expression are unclear (McConnachie L 2004). This is typically difficult or impossible to address with other approaches used today in genetics. It is this complete range of issues to which human hepatocytes lend themselves (testing for gene variations, mRNA, protein level and function) that makes cryopreserved human hepatocytes a promising resource for future research aimed at identifying and defining genetic variation(s) producing functional changes. Even today nearly half of all CYP alleles are of undefined functional relevance (Ingelman-Sundberg M 2005) because the means for defining their functional relevance has not been possible or is extremely laborious. As efforts to understand the relevance of genetic variation in the human genome increases, more efficient methods to evaluate and address alleles with functional relevance are essential. It is hoped this work serves as a stimulus for that future research.
CONCLUSIONS

Human hepatocytes are a potentially valuable resource for genomic research. Considerable research in the last 4 decades has already gone into testing and evaluating methods to most optimally cryopreserve human hepatic tissue. This commercial tissue should now be seriously considered for its potential to identify and characterize dysfunctional alleles. Integrating genomics, proteomics, proteomic function and gene expression profiling together with carefully cryopreserved human tissue may provide the critical information needed to identify the presence of new alleles and the likely mechanism by which such alleles affect gene expression. Linking alleles with their functional effects remains critical to the eventual clinical application of genomic information.
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FOOTNOTES

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FIGURE LEGENDS:

Figure 1. Histogram of 51 human hepatocyte lots based on their CYP2D6 activity and divided into three groups based on the predicted activity from each lot CYP2D6 genotype. Cross-hatched bars are hepatocytes with no functional genes for CYP2D6 (*3/*4 or *4/*4) genotype (far left bar in figure). Gray bars represent hepatocytes with at least one inactive gene (*4/*1; *4/*2; *3/*1, etc, or two alleles with diminished activity (*41/*10, *10/*10) and the remaining stippled black bars represent hepatocytes with two functional CYP2D6 alleles (*1,*2, *35) or one functional and one allele with only somewhat diminished activity (*1/*10, *2/*41, *35/*9, etc.). Where predicted and observed enzyme activity did not match they were marked by an asterisk. The CYP2D6*1 allele is the default wild type allele that is assumed to be present when tests for all other alleles are negative.

Figure 2A. Immunoblot of CYP2D6 levels from hepatocyte lots with no CYP2D6 activity (the asterisk sample (PFM; CYP2D6*10/*1 and WWM; CYP2D6*4/*2) indicate PFM has a normal level of CYP2D6 whereas WWM does not express this enzyme. These results suggest the allele in sample WWM is not a functional CYP2D6*2 allele.

2B. Results from the analysis of CYP2A6 expression, activity and genotype. The immunoblot shows the lot WWM is not devoid of all CYP protein, but has normal amounts of CYP2A6. Samples marked EVY and RML marked by an asterisk in figure 4 would be candidates for further study since their CYP2A6 genotypes, enzyme activities and immunodetectable protein levels suggest there is/are one or more null alleles present in these samples. Samples deficient in one CYP enzyme were not deficient in other CYP enzymes, suggesting low enzyme expression is not a general phenomenon in these hepatocyte lots.
**Figure 3A.** Gene sequence in exon 7 for *CYP2D6*56 allele. The thymine nucleotide at position 3201 marked with an asterisk is Cytosine in the wild type *CYP2D6* gene. The 3201C→T changes the codon CGA (Arg344)→TGA (Stop) and terminates reading of the message. The nucleotides underneath and backwards are the anti-sense sequence corresponding to this sense sequence.

**3B.** Sequence in exon 7 and site of the 3202C→T variation. The region is bold lettering corresponds to the region of the sequence provided in figure 3A.
### Table 2A

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1 pmol/min/10^6 cells ← 2nd activity assessment

### Table 2B

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20 pmol/min/10^6 cells ← 2nd activity assessment