Research Article

Genotyping of *CYP2D6* Polymorphisms by MALDI-TOF Mass Spectrometry in Sardinian People

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The *CYP2D6* enzyme is involved in the metabolism of many commonly prescribed drugs. The presence of *CYP2D6* gene SNPs can alter *CYP2D6* enzymatic activity with effects ranging considerably within a population. *Objectives.* In this study, we have developed a genotyping platform able to determine the alleles related to interindividual variability in the *CYP2D6* gene. *Design and Methods.* We used a long PCR strategy coupled to MALDI-TOF mass spectrometry (Sequenom) to develop a SNPs genotyping method. Furthermore, an amplification allele specific was carried out to infer the correct allelic phase. *Results.* We tested the multiplex platform in 250 DNA Sardinian samples and found it to be 100% concordant with the sequencing results of our previous work. *Conclusions.* The MALDI-TOF-based multiplexing system allowed simultaneous and efficient genotyping of a set of *CYP2D6* SNPs, evidencing its potential use in diagnostic test development to predict drug responses and clinical outcomes.

1. Introduction

The presence of polymorphisms in the cytochrome P450 2D6 (*CYP2D6*) gene may modulate enzyme levels affecting individual responses to pharmacological treatment in drug level, response, and adverse reactions and includes individuals with ultrarapid (UM), extensive (EM), intermediate (IM), and poor (PM) metabolizer status. Furthermore, the presence of multiple functional gene copies or the deletion of the entire gene results in increased or absent drug metabolism, respectively [1, 2]. Genotypic analysis to identify individual polymorphisms has become increasingly important during drug development and for selection of individualized therapies. For this reason, high-throughput technology for rapid, accurate, and efficient genotyping is needed. Several strategies and methods for SNPs genotyping have been developed. Techniques commonly used, such as PCR-RFLP, real-time PCR, and the TaqMan allele-specific assays from Applied Biosystems (CA, USA), are often laborious, and a restricted

number of alleles can be simultaneously detected by these techniques. Conversely, the high-throughput oligonucleotide microarray technology, such as the Affymetrix/Roche (CA, USA) AmpliChip CYP450 GeneChip test, has the disadvantage over other assays in that it cannot be customized by the user, and the benefits of this technology are often not compensable due to unfavourable costs [3]. In our previous work [4], we tried to create a complete genotyping platform for the simultaneous analysis of *CYP3A4*, *CYP3A5*, *CYP2C9*, *CYP2C19*, and *CYP2D6* SNPs. The genotyping of the *CYP2D6* gene was difficult due to its polymorphic nature, the presence of two flanking pseudogenes and copy number variants. To avoid false genotyping, resulting from nonspecific coamplification of the highly homologous pseudogenes, we developed the analysis of this gene in another way by using long PCR protocol coupled with single allele analysis and followed by sequencing [5]. In this work, we aimed to create a *CYP2D6* medium-throughput SNPs screening platform using the Sequenom (CA, USA) matrix-assisted

TABLE 1: List of 69 analyzed SNPs and correlation to aminoacid changes or transcriptional modifications.

CYP2D6 SNPs	Variations
-1584C>G	—
-1426C>T	—
-1235A>G	—
-1000G>A	—
-948C>A	—
-750_-749delGA	—
-740C>T	—
-678G>A	—
19G>A	V7M
31G>A	V11M
77G>A	R26H
82C>T	—
100C>T	P34S
124G>A	G42R
137_138insT	Frameshift
214G>C (*)	—
310G>T	—
746C>G	—
843T>G	—
883G>C	Splicing defect
957C>T	A85V
974C>A	L91M
984A>G	H94R
997C>G	—
1039C>T	—
1513C>T	—
1659G>A	V136I
1661G>C	—
1704C>G	Q151E
1707delT	Frameshift
1724C>T	—
1749A>G	N166D
1757C>T	—
1758G>A	G169R
1758G>T	G169X
1846G>A	Splicing defect
1863_1864insTTTCGCCCC	174_175insFRP
1869T>C	—
1943G>A	R201H
1979T>C	—
2291G>A	—
2483G>T	A237S
2539_2542delAACT	Frameshift
2549delA	Frameshift
2575C>A	—
2587_2590delGACT	Frameshift
2615_2617delAAG	K281del
2850C>T	R296C

TABLE 1: Continued.

CYP2D6 SNPs	Variations
2853A>C	I297L
2935A>C	H324P
2939G>A	—
2988G>A	Splicing defect
3176C>T	—
3183G>A	V338M
3198C>G	R343G
3277T>C	I369T
3288G>A	—
3384A>C	—
3582A>G	—
3584G>A	—
3790C>T	—
3828G>A	—
3853G>A	E410K
3877G>A	E418K
3948T>G	—
4115C>T	—
4155C>T (*)	H478Y
4180G>C	S486T
4401C>T	—
4481G>A	—

In MALDI-TOF MS analysis, (*) 214G>C SNP was used to discriminate gene conversion to CYP2D7P in Intron 1, and (°) 4155C>T SNP was used to discriminate gene conversion to CYP2D7P in Exon 9. A = alanine; R = arginine; N = asparagine; D = aspartic acid; C = cysteine; E = glutamic acid; Q = glutamine; G = glycine; H = histidine; I = isoleucine; L = leucine; K = lysine; M = methionine; F = phenylalanine; P = proline; S = serine; T = threonine; W = tryptophan; Y = tyrosine; V = valine. In bold characters newly discovered SNPs in [5, 17, 18, 21].

laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) [6], a widely used technology that is proving to be a competitive analysis method in SNPs genotyping. Advantages of MALDI-TOF MS over previously described methods include the option for medium-high-throughput automated analysis of SNPs, the relative ease of setup for multiplex assays, and the reduced costs per genotype [3].

2. Methods and Materials

SNPs and Sequences Selection. The 69 polymorphisms analyzed in this study were selected using principal SNP web databases such as the Human CYP Allele Nomenclature Committee [7] and the NCBI Single Nucleotide Polymorphism dbSNP [8] websites. Selection criteria depended mainly on the pharmacogenetic effects described for every allele in the Caucasian population [5, 7, 9–16]. Not all selected SNPs are involved in aminoacidic or transcriptional variation (Table 1). Some of these are silent or promoter, leader, trailer, and intronic changes and inserted in our study because they are essential for the reconstruction of haplotype phases. The SNPs' recombination allowed the reconstruction of 66 among

TABLE 2: List of primers used in *CYP2D6* long PCR protocols. The 5' 10-mer tag was added to PCR primers, in order to improve PCR efficiency.

Nucleotides position	Name	5' 10-mer tag	5'-Sequence-3'	Direction
From -1780 to -1758	P-1780 [5]	ACGTTGGATG	GTCCTCCTGTCCTCAGTGGAT	Forward
From -1584 to -1559	P-1584.WT [5]	ACGTTGGATG	CAGCCTGGACAACCTTGAAGAAGCC	Forward
From -1584 to -1559	P-1584.MUT [5]	ACGTTGGATG	CAGCCTGGACAACCTTGAAGAAGCG	Forward
From 4706 to 4728	2D6-R [19]	ACGTTGGATG	ACTGAGCCCTGGGAGGTAGTA	Reverse

allelic variants and subvariants. List of alleles is summarized in Table 5.

DNA Samples. In this study, we reanalyzed the genomic DNAs studied in [5]. Sardinian DNA samples were gently furnished by Professor Francesco Cucca, INN-CNR Cagliari Director. All participating individuals provided informed consent to genetic test. To genotype these samples, we implemented a *CYP2D6* Genotyping Platform based on MALDI-TOF MS. This way, we would be able to compare our genotyping results with a previous sequencing analysis in [5].

2.1. *CYP2D6* Genotyping Platform

2.1.1. Long Primary PCR. Selective amplification of the *CYP2D6* gene was carried out modifying a long PCR protocol implemented in our previous work [5]. Forward primer (P-1780, Table 2) was designed in a highly nonhomologous *CYP2D6/CYP2D7P/CYP2D8P* 5' untranslated region [5]. The reverse primer used was 2D6-R (Table 2), as previously described [19]. A 5' 10-mer tag (5'-ACGTTGGATG-3') was added to both PCR primers in order to improve PCR efficiency. PCR reactions were performed in a final volume of 5 μ L using the QIAGEN (Hilden, Germany) LongRange PCR Kit protocol [20] with the following minor modifications: 20 ng genomic DNA, 400 μ M of each PCR primer (Metabion, Martinsried, Germany), 0.2 U QIAGEN LongRange PCR enzyme, 1X QIAGEN LongRange PCR buffer (containing $MgCl_2$ 25 mM), and 500 μ M Invitrogen (CA, USA) 2'-deoxynucleoside-5'-triphosphate (dNTP) Set PCR Grade. The PCR conditions were as follows: initial denaturation at 93°C for 3 min; 35 cycles at 93°C for 30 s, 61°C for 30 s, and 68°C for 6 min.

2.1.2. SAP Dephosphorylation. To neutralize unincorporated dNTPs after amplification reactions, 0.3 U of Shrimp Alkaline Phosphatase (SAP) (Sequenom) [26, 27] was used. SAP cleaved a phosphate from the unincorporated dNTPs, converting them to 2'-deoxynucleoside-5'-diphosphate (dNDP) and rendering them unavailable for following reactions. Dephosphorylation conditions were as follows: 37°C for 20 min and 85°C for 5 min.

2.1.3. Assay Design. SNP-specific unextended minisequencing primers (UEPs) and multiplexed UEPs assays were designed using both the Sequenom MassARRAY assay design version 3.1 and the RealSNP assay database [28]. A sequence of 400 base pairs (bp) flanking each selected SNP was downloaded from the corresponding genomic sequence stored in

the public NCBI Single Nucleotide Polymorphism dbSNP database [8] or the Ensembl Genome Browser [29] and was analyzed by Vector NTI Suite Software version 5.5 (InforMax, Oxford, UK). Combination of the UEPs into multiplex assays was supported by these software applications to allow the optimization of several different parameters, for example, to avoid the risk of primer-primer interactions and hairpin-loop formations, GC content, molecular mass range, and annealing temperatures. To achieve the highest possible multiplexing levels, we tested many primer combinations, leading us to the final assay design consisting in a total of 69 SNPs successfully assembled in five medium-plex assays (13-, 14-, 13-, 14-, and 15-plex) (Table 3). For some SNPs, Sequenom MassARRAY assay design and RealSNP assay database could not design SNP-specific UEPs because of the presence of proximal SNPs. Other SNPs were excluded during platform validation because of UEPs cross-hybridization in highly homologous PCR template regions or for the presence of primer dimers which created false allele. But the high number of SNPs inserted in our analysis has allowed us to infer the correct alleles for each DNA analysis. List of SNPs not included is summarized in Table 4.

2.1.4. iPLEX Reactions. iPLEX reactions were carried out following the Sequenom standard low/medium-plex protocol [6, 26, 27], with minor modifications. Because of the length of the primary PCR fragment and the high GC content, we increased the denaturation time at 94°C from 5 to 30 sec and the annealing temperature from 52 to 56°C. An iPLEX reaction cocktail was added to the amplification products and thermocycled to process the iPLEX reaction, which involved the enzymatic addition of one of the four mass-modified nucleotides, 2',3'-dideoxynucleoside-5'-triphosphate (ddNTP), into the polymorphic site. During the iPLEX reaction, each primer was extended by one of the ddNTPs which terminated the extension of primers, thus producing allele-specific extension products of different masses. The iPLEX reaction cocktail included 0.222X iPLEX buffer plus, 0.5X iPLEX termination mix, 0.5X iPLEX enzyme, and UEPs, which were divided into four mass groups, according to the position of their respective mass peaks to use the whole spectrum (final concentrations of 0.8–2.0 μ M). In the iPLEX termination mix, all four ddNTPs are present at the same concentration. The reactions were performed using a two cycling loop program: initial denaturation at 94°C for 30 s; followed by 40 cycles of 94°C for 30 s, 56°C for 5 s, and 80°C for 5 s. This annealing and extension procedure was repeated four times (to give a total of 200 short cycles), followed by a final extension step of 3 min at 72°C. After desalting

TABLE 3: Sequences and molecular weights of unextended (UEP) and extended (EP) iPLEX primers.

UEPs' sequences			UD	UM	EP lower mass sequences		ELM	ELC	EP higher mass sequences		EHM	EHC
(1) 13- PLEX												
3877G>A	CCTTCCGCTTCCACCCC	F	4978		CCTTCCGCTTCCACCCCA		5249	A	CCTTCCGCTTCCACCCCG		5265	G
3183G>A	CCGCACCTGGCCCTATCA	R	5051		CCGCACCTGGCCCTATCAC		5299	G	CCGCACCTGOCCTATCAT		5378	A
2615_2617 delAAG	GGCAGCCACTCTCACCT	R	5091		GGCAGCCACTCTCACCTC		5339	del	GGCAGCCACTCTCACCTT		5418	AGA
2483G>T	GCGTAGGACCTTGCCAG	R	5211		GCGTAGGACCTTGCCAGC		5459	G	GCGTAGGACCTTGCCAGA		5483	T
3853G>A	GAAGCGGAAGGGCTTCT	R	5275		GAAGCGGAAGGGCTTCTC		5523	G	GAGCGGAAGGGCTTCTT		5603	A
3277T>C	TGCAGCGCTTTGGGGACA	F	5556		TGCAGCGCTTTGGGGACAC		5803	C	TGCAGCGCTTTGGGGACAT		5883	T
4180G>C	AGCTCATAGGGGGATGGG	R	5645		AGCTCATAGGGGGATGGGC		5892	G	AGCTCATAGGGGGATGGGG		5932	C
2939G>A	TGGGCTCACGCTGCACATC	R	5766		TGGGCTCACGCTGCACATCC		6013	G	TGGGCTCACGCTGCACATCT		6093	A
3288G>A	GATGTCATATGGGTTCACAC	R	5828		GATGTCATATGGGTTCACACC		6075	G	GATGTCATATGGGTTCACACT		6155	A
3176C>T	GGCCGTGTCCAACAGGAGAT	F	6167		GGCCGTGTCCAACAGGAGATC		6414	C	GGCCGTGTCCAACAGGAGATT		6494	T
2850C>T	AGCTTCAATGATGAGAACCTG	F	6454		AGCTTCAATGATGAGAACCTGC		6701	C	AGCTTCAATGATGAGAACCTGT		6781	T
2935A>C	AGTGCTCAGGCTGCACATCCGGA	R	7010		AGTGCTCAGGCTGCACATCCGGAG		7297	C	AGTGCTCAGGCTGCACATCCGGAT		7337	A
1757C>T	TTGTGCGCGCTTCGCCAACCACTC	F	7201		TTGTGCGCGCTTCGCCAACCACTCC		7448	C	TTGTGCGCGCTTCGCCAACCACTCT		7528	T
(2) 14- PLEX												
1661G>C	CAGAGGGCGCTTCTCCGT	F	5162		CAGAGGGCGCTTCTCCGTC		5410	C	CAGAGGGCGCTTCTCCGTG		5450	G
1869T>C	ACGGCTTTGTCCAAGAG	R	5210		ACGGCTTTGTCCAAGAGA		5482	T	ACGGCTTTGTCCAAGAGG		5498	C
1846G>A	GGGCGGAAAGGGGGCTC	R	5342		GGGCGGAAAGGGGGCTCC		5588	G	GGGCGGAAAGGGGGCTCT		5668	A
214G>C	TGGAGGGCGGCAGAGGT	F	5357		TGGAGGGCGGCAGAGGTC		5604	C	TGGAGGGCGGCAGAGGTG		5644	G
19G>A	CTGGGGCTAGAAGCACTG	F	5565		CTGGGGCTAGAAGCACTGA		5836	A	CTGGGGCTAGAAGCACTGG		5852	G
1707delT	GCGGCCTCTCGGTACACC	R	5702		GCGGCCTCTCGGTACACC		5949	del	GCGGCCTCTCGGTACACCA		5973	T
746C>G	GCGACCCAGCTCTCTGATC	R	5710		GCGACCCAGCTCTCTGATCC		5957	G	GCGACCCAGCTCTCTGATCG		5997	C
997C>G	GAACAGGGGGGGGTGCGC	R	5920		GAACAGGGGGGGGTGCGGC		6167	G	GAACAGGGGGGGGTGCGGG		6207	C
100C>T	AATGCTGGGTGCACGCTAC	F	6118		AATGCTGGGTGCACGCTTACC		6365	C	AATGCTGGGTGCACGCTACT		6445	T
1979T>C	GGACAGCCGACTCTCTCTC	R	6303		GGACAGCCGACTCTCTCTTCA		6574	T	GGACAGCCGACTCTCTCTTCG		6590	C
-948C>A	CAGGCTGGGGCAAGGGCTTTC	F	6488		CAGGCTGGGGCAAGGGCTTTCC		6735	C	CAGGCTGGGGCAAGGGCTTCA		6759	A
1749A>G	GCCCATCACCCACGGAGTGGT	R	6681		GCCCATCACCCACGGAGTGGTC		6929	G	GCCCATCACCCACGGAGTGGTT		7008	A
77G>A	GTAGCGTGCAGCCACGCTTGG	R	6792		GTAGCGTGCAGCCACGCTTGGC		7040	G	GTAGCGTGCAGCCACGCTTGGT		7120	A
1513C>T	AGCTGGACAGAGCCAGGGACTG	F	6835		AGCTGGACAGAGCCAGGGACTGC		7082	C	AGCTGGACAGAGCCAGGGACTGT		7162	T
(3) 13- PLEX												
984A>G	GCGAGTGTCTCTCGCG	R	4874		GCGAGTGTCTCTCGCCGC		5121	G	GCGAGTGTCTCTCGCCGT		5201	A
1863_1864ins TTTCGCCCC	GACGCCCTTTTCGCCCC	F	5043		GACGCCCTTTTCGCCCCA		5315	-	GACGCCCTTTTCGCCCCCT		5370	TTTCGCCCC
1039C>T	ACCCAGATCCTGGGTTT	F	5161		ACCCAGATCCTGGGTTTC		5409	C	ACCCAGATCCTGGGTTT		5489	T
1943G>A	CCAGCAGCCTGAGGAAG	R	5229		CCAGCAGCCTGAGGAAGC		5477	G	CCAGCAGCCTGAGGAAGT		5557	A
974C>A	CGGCCGTGGCGGAGGCG	F	5253		CGGCCGTGGCGGAGGCGC		5501	C	CGGCCGTGGCGGAGGCGA		5525	A
1704C>G	GGCAAGAAAGTCGCTGGAG	F	5614		GGCAAGAAAGTCGCTGGAGC		5861	C	GGCAAGAAAGTCGCTGGAGG		5901	G
310G>T	TAAATGCCCTTCTCCAGGA	R	5748		TAAATGCCCTTCTCCAGGAC		5995	G	TAAATGCCCTTCTCCAGGAA		6019	T
957C>T	GTGCTGCTCAATGGGCTGG	F	5876		GTGCTGCTCAATGGGCTGGC		6123	C	GTGCTGCTCAATGGGCTGGT		6203	T
883G>C	TCCCGAAGCGGCGCCGAA	R	6073		TCCCGAAGCGGCGCCGCAAC		6320	G	TCCCGAAGCGGCGCCGCAAG		6360	C
1659G>A	CGCGAGCAGAGGCGCTTCTCC	F	6408		CGCGAGCAGAGGCGCTTCTCCA		6679	A	CGCGAGCAGAGGCGCTTCTCCG		6695	G
31G>A	AGCAGGAAGATGGCACTATCA	R	6777		AGCAGGAAGATGGCACTATCAC		7024	G	AGCAGGAAGATGGCCACTATCAT		7104	A
1724C>T	GCGAAGGGCGCAAAAGCAGGC	R	7158		GCGAAGGGCGCAAAAGCAGGCA		7429	T	GCGAAGGGCGCAAAAGCAGGCG		7445	C
124G>A	TCACATGCAGCAGGTTGCCAGCC	R	7299		TCACATGCAGCAGGTTGCCAGCCC		7546	G	TCACATGCAGCAGGTTGCCAGCCT		7626	A

TABLE 3: Continued.

	UEP's sequences	UD	UM	EP lower mass sequences	ELM	ELC	EP higher mass sequences	EHM	EHC
(4) 14- PLEX									
3198C>G	ACCCATCTCTGGTCGGC	R	5082	ACCCATCTCTGGTCGGCC	5330	G	ACCCATCTCTGGTCGGCG	5370	C
2549delA	TGGGTCCCAGGTCAATCC	R	5162	TGGGTCCCAGGTCAATCCG	5450	del	TGGGTCCCAGGTCAATCCT	5490	A
3582A>G	GAATCTTGGAGGACCCA	F	5259	GAATCTTGGAGGACCCAA	5531	A	GAATCTTGGAGGACCCAG	5547	G
2539_2542 delAACT	ACAGCTGGATGAGTGCT	F	5540	ACAGCTGGATGAGTGCTA	5811	AACT	ACAGCTGGATGAGTGCTG	5827	del
3790C>T	CCACTCTCACCTGCATCT	F	5620	CCACTCTCACCTGCATCTC	5867	C	CCACTCTCACCTGCATCTT	5947	T
4115C>T	ACCTCCCTGCTGCAGCACTT	F	5989	ACCTCCCTGCTGCAGCACTTC	6236	C	ACCTCCCTGCTGCAGCACTTT	6316	T
4481G>A	GAATCTGACTGCCAGATTG	F	6117	GAATCTGACTGCCAGATTGA	6388	A	GAATCTGACTGCCAGATTGG	6404	G
3828G>A	ACTCATCACCAACTGTATC	F	6270	ACTCATCACCAACTGTATCA	6541	A	ACTCATCACCAACTGTATCG	6557	G
3384A>C	CATGCTGGGGTATCACAGG	R	6447	CATGCTGGGGTATCACAGGG	6734	C	CATGCTGGGGTATCACAGGT	6774	A
2853A>C	GAGAACAGGTGAGCCACACTA	R	6722	GAGAACAGGTGAGCCACACTAG	7010	C	GAGAACAGGTGAGCCACACTAT	7050	A
2988G>A	CATGTGCCCCGCTGTACCCCTT	R	6888	CATGTGCCCCGCTGTACCCCTTC	7135	G	CATGTGCCCCGCTGTACCCCTTT	7215	A
3584G>A	GTCAGAAATGTTGGAGGACCCAAC	F	7098	GTCAGAAATGTTGGAGGACCCAAC	7369	A	GTCAGAAATGTTGGAGGACCCAACG	7385	G
4401C>T	TAACTGACATCTGCTCAGCCTCAAC	R	7546	TAACTGACATCTGCTCAGCCTCAACA	7817	T	TAACTGACATCTGCTCAGCCTCAACG	7833	C
2587_2590 delGACT	ATCAGCTCAGCCCCCGGAGACCTGA	F	7846	ATCAGCTCAGCCCCCGGAGACCTGAC	8093	GACT	ATCAGCTCAGCCCCCGGAGACCTGAG	8133	del
(5) 15- PLEX									
3948T>G	TCTCAGCAGGTGCCTG	F	4873	TCTCAGCAGGTGCCTGG	5160	G	TCTCAGCAGGTGCCTT	5200	T
-1235A>G	GCACCAACCCAGCCTAAT	R	5084	GCACCAACCCAGCCTAATC	5332	G	GCACCAACCCAGCCTAATT	5411	A
137_138insT	GAAGTCCACATGCAGCA	R	5188	GAAGTCCACATGCAGCAA	5460	T	GAAGTCCACATGCAGCAG	5476	—
4155C>T	CAGCCCGGCCAGCCAC	F	5376	CAGCCCGGCCAGCCACC	5623	C	CAGCCCGGCCAGCCACT	5703	T
82C>T	GTGTAGCGTGCAGCCAGC	R	5830	GTGTAGCGTGCAGCCAGCA	6101	T	GTGTAGCGTGCAGCCAGCG	6117	C
-1584C>G	CTGGACAACTTGGAAAGAAC	F	6135	CTGGACAACTTGGAAAGAACCC	6382	C	CTGGACAACTTGGAAAGAACCG	6422	G
2575C>A	GACCTGGGACCCAGCCAGCC	F	6362	GACCTGGGACCCAGCCAGCCC	6609	C	GACCTGGGACCCAGCCAGCCA	6633	A
843T>G	ACTAGGACCTGTAGTCTGGG	F	6502	ACTAGGACCTGTAGTCTGGGG	6789	G	ACTAGGACCTGTAGTCTGGGGT	6829	T
-740C>T	ACAGACTCACACTGACACTTAG	R	6672	ACAGACTCACACTGACACTTAGA	6944	T	ACAGACTCACACTGACACTTAGG	6960	C
-678G>A	CTTTGTGTGGGTGATTTTCTGC	F	6769	CTTTGTGTGGGTGATTTTCTGCA	7041	A	CTTTGTGTGGGTGATTTTCTGCG	7057	G
-750_-749 delGA	TGTGACTGGTGTGTGTGAGAGA	F	6902	TGTGACTGGTGTGTGTGAGAGAA	7173	del	TGTGACTGGTGTGTGTGAGAGAG	7189	GA
2291G>A	CACCTGCCAAAGTCCAGCCTCCAC	R	7204	CACCTGCCAAAGTCCAGCCTCCACC	7451	G	CACCTGCCAAAGTCCAGCCTCCACT	7531	A
-1426C>T	GTGTGCCAACACGCTAGCTTTT	R	7286	GTGTGCCAACACGCTAGCTTTTAA	7533	T	GTGTGCCAACACGCTAGCTTTTG	7573	C
1758G>T>A	TTGTGCGCGCTTTCGCCAACCACTCC	F	7490	TTGTGCGCGCTTTCGCCAACCACTCCA	7761	A	TTGTGCGCGCTTTCGCCAACCACTCCT	7777	G
-1000G>A	ACATCCTCCCGGGTGCCTGAGGGT	R	7635	ACATCCTCCCGGGTGCCTGAGGGTC	7882	G	ACATCCTCCCGGGTGCCTGAGGGTT	7962	A

UD: Unextended mini-sequencing primer Direction; F: Forward UEP direction; R: Reverse UEP direction; UM: Unextended mini-sequencing primers Mass; ELM: Extended primer Lower Mass; EHM: Extended primer Higher Mass; ELC: Extended primer lower Mass nucleotide Called; EHC: Extended primer higher Mass nucleotide Called; All molecular weight masses are expressed in Dalton (Da).

TABLE 4: List of SNPs excluded in assay design validation and correlation to transcriptional variations.

SNPs excluded	Variations	Reason
-1770G>A	—	Cross-hybridization
-1298G>A	—	Primer dimers
-1253A>G	—	Primer dimers
-1237_-1236insAA	—	Proximal SNP -1235A>G Primer dimers
1973_1974insG	Frameshift	Proximal SNP 1979T>C
1976G>A	—	Proximal SNP 1979T>C
1978C>T	—	Proximal SNP 1979T>C
2097A>G	—	Primer dimers
2470T>C	—	Cross hybridization
4042G>A	—	Primer dimers

by addition of 6 mg clean resin (Sequenom), each 384-well sample was diluted with 16 μL of sterilized $\text{H}_2\text{O}_{\text{dd}}$.

Multiplex PCR reactions, SAP dephosphorylation, and iPLEX reactions were performed using Thermo-Fast 384 PCR Plates (ABgene, Epsom, UK) and a DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad, CA, USA). All pipetting steps were performed using the automatic station Matrix PlateMate 2 $\times$ 2 (Sequenom).

2.1.5. MALDI-TOF MS Measurement. An aliquot ranging from 15 to 20 nL of each iPLEX reaction product was loaded in a 384-spot SpectroChip (Sequenom) using the MassArray Nanodispenser (Samsung, Seoul, Republic of Korea). SpectroChip analysis was performed by MassARRAY Compact System (Sequenom). After laser desorption/ionization, automated spectra acquisition analysis was performed and interpreted using Sequenom MassARRAY RT version 3.3 software. Examples of multiplex mass spectrum and cluster plot distributions are shown in Figures 1 and 2.

2.2. CYP2D6 Single Allele Genotyping. Following direction of our previous work [5], we decided to apply the single allele protocol creating a single allele genotyping method MALDI-TOF MS based. For each sample, a double long PCR reaction was carried out using P-1584_WT or P-1584_MUT [5] (Table 2) as forward primers. The reverse primer was 2D6-R [19] for both PCR reactions. In this way, we were able to directly determine a direct and correct chromosome phase in samples presenting with a heterozygous status for -1584G>C SNP. PCR reactions were performed in a final volume of 5 μL using 20 ng genomic DNA, 400 μM of each PCR primer (Metabion), 0.2 U QIAGEN LongRange PCR enzyme, 1X QIAGEN LongRange PCR buffer (containing MgCl_2 25 mM), and 800 μM Invitrogen dNTP set PCR grade. The PCR conditions were as follows: initial denaturation at 93°C for 3 min; 10 cycles at 93°C for 30 s, 67°C for 30 s, and 68°C for 3 min; 25 cycles at 93°C for 30 s, 65°C for 30 s, and 68°C for 6 min. SAP dephosphorylation, iPLEX Reactions and MALDI-TOF MS measurement were performed without

TABLE 5: CYP2D6 allele frequencies in 250 healthy Sardinian people. Total chromosomes number = 500. Human cytochrome P450 Allele Nomenclature Committee [7] served as reference for variant allele and correlated enzymatic activity.

Variant allele	Number of chromosomes	Frequency (%)	Correlated enzymatic activity
*1A	148	29.6	EM
*1B	5	1.0	EM
*1D	4	0.8	EM
*1E	—	—	EM
*2A	75	15.0	EM
*2B	—	—	EM
*2D	—	—	EM
*2E	—	—	EM
*2F	—	—	EM
*2G	—	—	EM
*2K	—	—	EM
*2L	11	2.2	EM
*2M	8	1.6	EM
SH3	7	1.4	Not known
SH4	1	0.2	Not known
*3A	—	—	PM
*3B	11	2.2	PM
*4A	84	16.8	PM
*4B	—	—	PM
*4D	—	—	PM
*4K	—	—	PM
*4L	—	—	PM
*4M	—	—	PM
*4N	—	—	PM
*5	5	1.0	PM
*6A	1	0.2	PM
*6C	—	—	PM
*6D	—	—	PM
*7	—	—	PM
*8	—	—	PM
*9	1	0.2	IM
*10A	—	—	IM
*10B	27	5.4	IM
*11	—	—	PM
*12	—	—	PM
*14A	—	—	PM
*14B	—	—	IM
*15	3	0.6	PM
*17	—	—	IM
*19	—	—	PM
*20	1	0.2	PM
*22	—	—	Not known
*23	—	—	Not known

TABLE 5: Continued.

Variant allele	Number of chromosomes	Frequency (%)	Correlated enzymatic activity
*24	—	—	Not known
*25	—	—	Not known
*26	—	—	Not known
*27	—	—	EM
*28	4	0.8	Not known
*29	—	—	IM
*30	—	—	Not known
*31	—	—	PM
*32	—	—	Not known
*33	—	—	EM
*35A	5	1.0	EM
*36	—	—	IM
*37	—	—	Not known
*38	—	—	PM
*39	—	—	EM
*41	46	9.2	IM
SH1	41	8.2	Not known
SH2	2	0.4	Not known
*43	—	—	Not known
*58	—	—	Not known
*59	—	—	IM
*64	—	—	Not known
*65	—	—	Not known
*1xN	4	0.8	UM
*2xN	6	1.2	UM

SH1,2,3,4 = Sardinian haplotype 1,2,3,4 [5, 22–25]. *CYP2D6**5, *1xN; and *2xN alleles were evaluated by The *CYP2D6* Applied Biosystems CNV Assay in [5].

modifications as indicated in paragraph 1 “*CYP2D6* Genotyping Platform”. In multiplexed assay 5, –1584G>C UEP (Table 3) was excluded. Examples of cluster distribution for novel 3176C>T and 3948T>G SNPs [5, 17, 18] are shown in Figure 3.

3. Results and Discussion

A *CYP2D6* screening assay was developed using the Sequenom MassARRAY platform to simultaneously identify the most frequent and some rare *CYP2D6* Caucasian alleles. We have modified the basic Sequenom iPLEX assay and used a new primary PCR strategy based on the amplification of the entire gene [5] coupled with multiplex primer extension reactions. This strategy avoids false genotyping, which would result in nonspecific coamplification of the homologous pseudogenes *CYP2D7P* and *CYP2D8P*, and, secondly, it reduces the number of PCR primers used to select regions containing the targeted polymorphisms. Multiplexing was performed for 69 SNPs, which represents 66 of the most frequent and some rarer variants and subvariants reported

FIGURE 1: An example of 15-plex mass spectrum is shown (Well 5). List of SNPs investigated in this plex is shown in Table 3. The figure shows two examples highlighted in different colours. Unextended primer (UEP) peak is marked by an asterisk and dotted arrow, while the solid arrows indicate the presence of the two different alleles. Dotted vertical lines represent UEPs and extended primers (EPs) expected masses. In blue, an AG heterozygous genotype example is shown. *: –1235A>G UEP; expected mass = 5084 Dalton (Da). G: –1235A>G EP; expected mass = 5332 Da. A: –1235A>G EP; expected mass = 5411 Da. In red, a CC *wild type* homozygous genotype example is shown. *: 82C>T UEP; expected mass = 5830 Da. T: 82C>T not EP; expected mass = 6101 Da. C: 82C>T EP; expected mass = 6117 Da.

to date in the Caucasian population [5, 7, 9–16] and known to be responsible for absent, reduced or extensive metabolic activity (Tables 1 and 5). Due to the high possibility of recombination, it was possible to insert African, African-American (*CYP2D6**2L, *2 M, *4 N, *64, and *65) and Asian (*CYP2D6**3A, *4B, *4L, *14, *36, and *39) variants in the study. A sample of 250 unrelated healthy Sardinian individuals analyzed in [5] was submitted to MALDI-TOF MS genotyping for these 69 *CYP2D6* SNPs. An example of multiplex mass spectrum is shown in Figure 1. In Figure 2(a), an example of the cluster plot distribution for the 1661G>C SNP is shown. Spectrum peak intensities were not correctly balanced in some heterozygous samples (Figures 2(c) and 2(d)), which appeared as outliers in the cluster distribution (Figure 2(a) point δ and ϵ). The *CYP2D6* Applied Biosystems Copy Number Variation (CNV) Assay used to analyze these DNAs in our previous work [5] detected the presence of duplications or multiplications in 100% of the analyzed samples presenting this kind of distribution. Furthermore, in samples presenting a heterozygous status for –1584G>C SNP, we applied long PCR single allele analysis [5] to MALDI-TOF MS screening assays and inferred a direct haplotype phase. To verify if our *CYP2D6* platform works correctly, we compared genotyping results and haplotype phase elaborated in the two screening platforms with our results found in [5]. A consensus of 100% was found for all samples (Table 5). Moreover, for all samples presenting the 214G>C SNP in homo- or heterozygous status in MALDI-TOF MS analysis, it was possible to verify in our previous sequencing analysis

FIGURE 2: In (a) a Cluster plot for 1661G>C SNP is shown. Homozygous wild type G/G samples are displayed in (α), homozygous mutant C/C samples in (β) and heterozygous G/C samples in (γ); in (δ) and (ε) are displayed outlier samples resulting in gene copy number variation [5]; (ζ) negative control (H2Odd). In (b) a Mass Spectra for the detection of 1661G>C in a heterozygous G/C sample, displayed in (a) position (γ). In (c) a Mass Spectra for the detection of 1661G>C in the outlier sample, displayed in (a) position (δ), and resulting in $CYP2D6^*1 \times N$ allele [5]. In (d) Mass Spectra for the detection of 1661G > C in an outlier sample, displayed in (a) position (ε), and resulting in $CYP2D6^*2 \times N$ allele [5].

[5] the presence of all SNPs correlated to gene conversion to *CYP2D7P* in Intron 1 (Figure 4), which indicated the reliability of MALDI-TOF analysis for this variation.

4. Conclusions

Differences in drug responses could be due to genetic factors. Knowledge of individual genetic profiling is clinically important and provides benefits for future medical care by predicting the drug response or developing DNA-based tests. Substantial interindividual variability in response to specific therapies might be caused by the presence of polymorphisms in genes encoding components of drug metabolism pathways, such as the CYP450 family genes. Polymorphisms in *CYP2D6* gene have been thoroughly investigated, including their associations with the incidence of adverse reactions. In this study,

we have developed a reliable medium-throughput genotyping platform using the Sequenom MassARRAY system to provide a simultaneous screening of the most relevant *CYP2D6* gene variants in several hundreds of subjects. MALDI-TOF MS-based analyses have the potential to become a useful approach in clinical diagnoses, as they are very flexible and applicable to individualized genetic therapies. The screening platform developed in this study, coupled with The *CYP2D6* Applied Biosystems CNV Assay, provides a robust alternative to many currently available CYP450-genotyping approaches and aims to increase the number of responders and to limit the incidences of adverse events. Moreover, by modifying long PCR Forward primer, we have implemented a rapid strategy to infer the phase by direct analysis using MALDI-TOS MS multiplex assays. Concordance between data found in this work and our direct genomic DNA sequencing analysis done in [5] reliably validated our MALDI-TOF MS-based platform.

FIGURE 3: MALDI-TOF MS cluster plot distribution for 3176C>T [17] and 3948G>T [18] newly discovered SNPs [5]. In each cluster, there are visualized 32 samples presenting heterozygous status for -1584G>C SNP and submitted to both types of PCR analysis, LongRange PCR (Section 2, paragraph 1) and the two single allele PCR (Section 2, paragraph 2), for a total of 96 PCR analyses. (a) Cluster plot for 3176C>T SNP. (α) homozygous *wild type* C/C samples; (β) one out of the two heterozygous C/T samples presenting heterozygous status for -1584G>C SNP also; (γ) the same heterozygous sample analysed in single allele PCR; (ζ) negative control (H_2O_{dd}). (b) Cluster plot for 3948T>G SNP. (α) homozygous *wild type* T/T samples; (β) the only heterozygous T/G sample; (γ) the only heterozygous sample analysed in single allele PCR; (ζ) negative control (H_2O_{dd}).

FIGURE 4: (a) Electropherograms of a homozygous *wild type* and (b) a homozygous mutate sample for *CYP2D6/CYP2D7P* gene conversion in Intron 1 analyzed in [5]. SNPs are circled in purple; reference positions are indicated under each SNP. For all samples presenting 214G>C SNP in homo- or heterozygous status in MALDI-TOF MS analysis, it was confirmed the presence of all SNPs correlated to gene conversion in Intron 1 by sequencing analysis.

Conflict of Interests

The authors declare not to have a financial relation with the commercial identities mentioned in the paper that might lead to a conflict of interests.

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