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Cytochrome P450 2D6 Variants in a Caucasian Population: Allele Frequencies and Phenotypic Consequences

Christoph Sachse, Jürgen Brockmöller, Steffen Bauer, and Ivar Roots

Institute of Clinical Pharmacology, University Clinic Charité, Humboldt University of Berlin, Berlin

Summary

Cytochrome P450 2D6 (CYP2D6) metabolizes many important drugs. CYP2D6 activity ranges from complete deficiency to ultrafast metabolism, depending on at least 16 different known alleles. Their frequencies were determined in 589 unrelated German volunteers and correlated with enzyme activity measured by phenotyping with dextromethorphan or debrisoquine. For genotyping, nested PCR-RFLP tests from a PCR amplificate of the entire *CYP2D6* gene were developed. The frequency of the *CYP2D6*1* allele coding for extensive metabolizer (EM) phenotype was .364. The alleles coding for slightly (*CYP2D6*2*) or moderately (**9* and **10*) reduced activity (intermediate metabolizer phenotype [IM]) showed frequencies of .324, .018, and .015, respectively. By use of novel PCR tests for discrimination, *CYP2D6* gene duplication alleles were found with frequencies of .005 (**1×2*), .013 (**2×2*), and .001 (**4×2*). Frequencies of alleles with complete deficiency (poor metabolizer phenotype [PM]) were .207 (**4*), .020 (**3* and **5*), .009 (**6*), and .001 (**7*, **15*, and **16*). The defective *CYP2D6* alleles **8*, **11*, **12*, **13*, and **14* were not found. All 41 PMs (7.0%) in this sample were explained by five mutations detected by four PCR-RFLP tests, which may suffice, together with the gene duplication test, for clinical prediction of *CYP2D6* capacity. Three novel variants of known *CYP2D6* alleles were discovered: **1C* ($T_{1957}C$), **2B* (additional $C_{2558}T$), and **4E* (additional $C_{2938}T$). Analysis of variance showed significant differences in enzymatic activity measured by the dextromethorphan metabolic ratio (MR) between carriers of EM/PM (mean MR = .006) and IM/PM (mean MR = .014) alleles and between carriers of one (mean MR = .009) and two (mean MR = .003) functional alleles. The results of this study provide a solid basis for prediction of CYP2D6 capacity, as required in drug research and routine drug treatment.

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Address for correspondence and reprints: Dr. Jürgen Brockmöller, Institut für Klinische Pharmakologie, Universitätsklinikum Charité der Humboldt-Universität zu Berlin, Schumannstrasse 20/21, D-10098 Berlin, Germany.

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Introduction

Cytochrome P450 2D6 (*CYP2D6*, debrisoquine/sparteine hydroxylase) may be involved in metabolism of ~25% of drugs (Benet et al. 1996). About 5%–10% of Caucasians are so-called poor metabolizers (PM) of debrisoquine, completely lacking CYP2D6 activity because of inactivating mutations in both alleles of the *CYP2D6* gene (location 22q13.1). People with active CYP2D6 show a broad spectrum of enzyme activity, from relatively low to ultra-high. In many instances, prediction of CYP2D6 activity is used to explain or to predict outliers in drug studies. Since therapeutic efficacy and adverse events in treatment with many drugs depend on CYP2D6 activity, it is anticipated that genotyping of *CYP2D6* may become a routine part of an individually optimized drug treatment.

At present, it is questionable which of the mutant alleles should routinely be identified to allow a sufficiently reliable but still practicable estimation of a person's metabolic capacity. In addition to the wild-type gene (*CYP2D6*1*), 15 different alleles of *CYP2D6* (some of them with subtypes), associated with deficient, reduced, or increased activity, are known in Caucasians. They are termed by the unified nomenclature developed by Daly et al. (1996*a*). Most alleles consist of point mutations, but also a deletion of the entire gene and gene conversions, resulting in "hybrid" alleles **13* and **16*, exist (Panserat et al. 1995; Daly et al. 1996*b*). There are gene duplications of the *2 allele (**2×2;* Johansson et al. 1993), as well as the **1* and **4* alleles (Masimirembwa et al. 1993; Dahl et al. 1995; Løvlie et al. 1996) and higher amplifications of *CYP2D6* genes (Johansson et al. 1993; Aklillu et al. 1996). The population frequency of mutant alleles is dependent on ethnic origin, as summarized by Bertilsson (1995). The most frequent inactivating mutation among Caucasians is the splice-site mutation $G_{1934}A$ defining the *CYP2D6*4* allele (former type B allele), which results in loss of enzyme activity. The allele frequency of **4* was found earlier to be between .1 and .2 among Caucasians; additional information is available about the allele frequencies of **3* (.007–.014), **5* (.010–.080), **6* (.013–.018), **7* (.015), and **9* (.015), with allele frequencies in parentheses representing the data from several studies in dif-

ferent European and White American groups (Broly et al. 1991; Daly et al. 1991; Tyndale et al. 1991; Evert et al. 1994*a*, 1994*b*; Saxena et al. 1994; Tefre et al. 1994; Daly et al. 1995). However, these allele frequency data were derived from different groups, which were sometimes small in sample size. For rare mutations, frequencies should be estimated in populations independent from those in which the mutations were originally discovered in order to exclude singular occurrence in local subgroups, which would make determination of such variants for clinical purposes unnecessary. This report presents a study in a large sample of the German population on the frequency of the known *CYP2D6* mutations, their allelic linkage, and their effects on enzyme activity. These data are required for many applications related to drug treatment.

Subjects, Material, and Methods

Subjects

Five hundred eighty-nine unrelated German individuals participated in the phenotyping and genotyping tests. They include 330 healthy volunteers and 259 patients with various diseases but without impairment in liver or kidney function and without known malignancy. All volunteers had given informed and written assent to genotyping and phenotyping, and both procedures were approved by the local Ethics Committee. Mean age was 40 years in males (range 19–90 years) and 48 years in female (range 21–91 years). Volunteers did not participate in drug trials for at least 4 wk, prior to testing, and they were informed that they must not drink quinine- or grapefruit-containing beverages.

CYP2D6 *Phenotyping Procedures*

All 589 volunteers were phenotyped for their CYP2D6 capacity, 456 with dextromethorphan, and 133 with debrisoquine. The dextromethorphan test was carried out with a single 30-mg test dose, a urine-collection period of 5 h, and quantification of dextromethorphan and the CYP2D6-catalyzed metabolite dextrorphan in urine by high-performance liquid chromtaography (HPLC) (Chen et al. 1990) to calculate the molar urinary metabolic ratio (MR). Detection limits were 10 ng/ml each for dextromethorphan and dextrorphan. The debrisoquine test was performed with 5 mg debrisoquine (5 h urine-sampling period). Debrisoquine and 4-hydroxydebrisoquine in urine were measured by gas chromatography according to the method of Lennard et al. (1977), and the molar MR was calculated. Detection limit was 10 ng/ml each for debrisoquine and 4-hydroxydebrisoquine.

DNA Isolation

Samples of 5–10 ml of whole EDTA blood were used, leukocytes were isolated, and DNA was extracted by phenol/chloroform (Sambrook et al. 1989). DNA was dissolved in 10 mM Tris-HCl pH 8.0, 1 mM EDTA, and stored at 4°C until RFLP and PCR analysis.

CYP2D6 *Genotype Determination*

We developed combined PCR-RFLP tests to detect the known point mutations of *CYP2D6* as summarized in table 1. The first step was the amplification of a 4,681-bp genomic DNA fragment containing all nine *CYP2D6* exons using the Expand Long Template PCR System™ (Boehringer). A 50-µl PCR mix contained 50 mM Tris-HCl pH 9.2, 1.75 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 135 µM dNTPs, 0.5 µM of each of the primers P100 and P200 (all primers are given in table 2), 4 U of enzyme mix (*Taq* and *Pwo* polymerases), and ~0.5 µg of genomic DNA. Thermocycling conditions (ABI GeneAmp 9600) were as follows: initial denaturation (2 min 94°C), 35 cycles of 10 s 93°C, 30 s 60°C, 5 min 68°C, and terminal extension (7 min 68°C). If PCR was successful (checked by 1% agarose gel electrophoresis), 45 µl of PCR product were diluted with 5 volumes of 10 mM Tris pH 8.0, 1 mM EDTA, and stored at 4°C. The diluted 4,681-bp PCR product was stable for ⩾6 mo, when stored at 4°C.

In the subsequent nested PCRs (table 1) (ABI GeneAmp PCR system 9600), conditions were as follows: reactions 1, 2, and 5–9 worked with initial denaturation for 2 min at 94°C, 25 PCR cycles of 30 s 94°C, 10 s 60°C, 1 min 72°C, and a terminal extension for 7 min at 72°C. A 50-µl PCR mix contained 10 mM Tris-HCl pH 8.3, 1.25 mM $MgCl_2$, 50 mM KCl, 200 µM dNTPs, 0.2 µM of each of the primers, 2.5 U *Taq* polymerase, and 1 µl of the diluted 4,681-bp PCR product.

PCR product 4 was amplified by 2 min at 94°C, 25 cycles of 30 s 94°C, 10 s 58°C, 1 min 72°C, and terminal extension for 7 min at 72°C. A 65-µl PCR mix contained 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 160 µM dNTPs, 0.1 µM of each of the primers, 2.5 U *Taq* polymerase, and 1 µl of the diluted 4,681-bp PCR product.

After amplification, the products of nested PCRs were analyzed by an 1% agarose gel electrophoresis and digested with the respective restriction endonucleases (New England Biolabs). The enzymes and restriction-fragment lengths for all the tests are given in table 1. The digestion products were analyzed on a 3% agarose gel, together with a 100-bp DNA molecular weight marker (Gibco BRL). Typical patterns of the wild-type, the heterozygous, and the homozygous mutant alleles are presented in figure 1.

CYP2D6 gene duplications were detected by *Xba*I RFLP (Skoda et al. 1988) and *Eco*RI RFLP of samples with 42-kb or 44-kb *Xba*I fragments (Johansson et al. 1993). In addition, a recently described PCR method for

Table 1

Design of the PCR Tests for *CYP2D6* Mutations

PCR No. and Detected Mutation	PCR-RFLP Conditions: PCR Primers	PCR-RFLP Conditions: Fragment Length (bp)	PCR-RFLP Conditions: Restriction Enzyme	Diagnostic Fragment Pattern (bp): Wild-Type Allele	Diagnostic Fragment Pattern (bp): Mutant Allele
1:					
Del T_{1795}	P*3/P2	353	*Bst*NI	190/163	190/139/23
$G_{1934}A$	...	...	...	190/163	353
$G_{1846}T/A$	...	...	*Msp*I	278/75	353
2 Del A_{2637}	P51/P522	201	*Bsa*AI	201	180/20
3[a] *5	P13/P24	3,500	...	None	3,500
4:					
$C_{188}T$	P11/P12	433	*Hph*I	362/71	262/100/71
$G_{212}A$	...	...	*Msp*I	242/141/50	242/191
Ins T_{226}	...	...	*Bsp*MI	157/156/120	278/156
5 $A_{3023}C$	P61/P*6	187	*Fok*I	124/26/37	124/63
6 $G_{971}C$	P21/P22	256	*Pst*I	114/106/36	142/114
7:					
*9	P*5/P62	386	*Bsp*MI	230/124/32	262/124
$C_{2938}T$	...	...	+*Mbo*II	230/124/32	354/32
8 $G_{4268}C$	P81/P92	881	*Ban*II	420/235/107/101/18	420/336/107/18
9[b] $G_{1749}C$	P31/P2	467	*Bsm*AI	311/156	209/156/102
10[c]:					
*13, *16	P71/P24	8,000	...	None	8,000
*5	...	9,500	...	None	9,500
11[c] *16	P161/P162	1,400	...	None	1,400
12[d] *CYP2D6*M×N*	P2x2f/P2x2r	10,000	...	None	10,000
12A *CYP2D6*1×N*	P2x2f/P92	264	*Ban*II	*1: 231/33	*2, *4: 264
12B *CYP2D6*4×N*	P11/P12	433	*Hph*I	*1, *2: 362/71	*4: 262/100/71

NOTE.—PCRs 1–2 and 4–9 are nested amplifications from the P100/P200-PCR product, 3 and 10–12 are amplifications from genomic DNA, and 12A and 12B are nested PCRs from the product of PCR 12.

[a] Steen et al. (1995).

[b] Not an essential reaction, but, together with results of reactions 4, 7, and 8, it served for validation of alleles *2, *4, and *10.

[c] Daly et al. (1996b).

[d] Johansson et al. (1996).

allele-specific detection of *CYP2D6* amplification was performed (Johansson et al. 1996). To distinguish between different types of allele duplication, two additional PCR-RFLP tests (reactions 12A and 12B) were developed, both as nested PCRs from the diluted PCR product of reaction 12, which is a 10-kb fragment between exon 9 of allele 1 and intron 2 of allele 2: a PCR-RFLP-test detecting the *2-related $G_{4268}C$ mutation (reaction 12A) showed whether *1 or *2 is duplicated (in *1×2/*2 or *2×2/*1 constellation). Primers P2×2f and P92 (table 2) were used (PCR conditions as for reaction 8) to amplify a 264-bp product, which was *Ban*II digested (see table 1; not shown as figure). Performing reaction 12B (same conditions as in reaction 4) from the 10-kb gene duplication product, detection of the *4-associated $C_{188}T$ mutation by *Hph*I digestion served as an indirect proof of a duplicated *4 allele in cases of a questionable *2×2/*4 or *4×2/*2 constellation.

For detection of *CYP2D6*5*, the method of Steen et al. (1995) was used in addition to *Xba*I RFLP (PCR No. 3 in table 1). Long distance PCR technique was applied (Boehringer Expand Long Template PCR System™) using the same concentrations of PCR compounds and PCR conditions as described above for the PCR reaction amplifying the entire *CYP2D6*.

Screening for *CYP2D6*13* and *CYP2D6*16* was performed using allele specific PCR tests as described by Daly et al. (1996*b*).

CYP2D6 *Sequence Analyses*

In selected samples, the whole *CYP2D6* gene, including all exons, splice site–flanking regions, and 5′- and 3′-flanking regions, were sequenced with a *Taq*-Dye-deoxy-terminator-cycle-sequencing kit (Applied Biosystems) and sequencing primers as described by Sachse et al. (1996). The resulting fragments were analyzed on an

Table 2

Sequences and Locations of Primers Used in PCR Reactions

Primer	Sequence	Orientation and Position[a]		
P100	5′ GGCCTACCCTGGGTAAGGGCCTGGAGCAGGA	f	−180	−150
P200	5′ CTCAGCCTCAACGTACCCCTGTCTCAAATGCG	r	+92	+123
P*3	5′ CCTGGGCAAGAAGTCGCTGGACCAG	f	1770	1794
P2	5′ GAGACTCCTCGGTCTCTCG	r	2104	2122
P51	5′ GCTGGGGCCTGAGACTT	f	2457	2473
P522	5′ GGCTGGGTCCCAGGTCATAC	r	2638	2657
P13	5′ ACCGGGCACCTGTACTCCTCA	f (*2D7*)	+1619	+1639
P24	5′ GCATGAGCTAAGGCACCCAGAC	r	+3444	+3465
P11	5′ TCAACACAGCAGGTTCA	f	−82	−66
P12	5′ CTGTGGTTTCACCCACC	r	335	351
P61	5′ CCCGTTCTGTCCCGAGTATG	f	2859	2878
P*6	5′ GGGCTCACGCTGCACATCAGGA	r	3024	3045
P21	5′ TGCTCACTCCTGGTAGCC	f	865	882
P22	5′ AGGATCTGGGTGATGGGCA	r	1102	1120
P*5	5′ AGGCCTTCCTGGCAGAGATGAAG	f	2680	2702
P62	5′ CCCCTGCACTGTTTCCCAGA	r	3047	3066
P81	5′ CGTCTAGTGGGGAGACAAAC	f	3621	3640
P92	5′ CTCAGCCTCAACGTACCCCT	r	+104	+123
P31	5′ TAATGCCTTCATGGCCACGCG	f	1651	1671
P71	5′ TCCGACCAGGCCTTTCTACCAC	f	−297	−276
P161	5′ TGGGGCCCAAGGCAGGGACT	f (*2D7*)	3020	3039
P162	5′ TGCTCAGCCTCAACGTACCCC	r	+105	+125
P2x2f	5′ GCCACCATGGTGTCTTTGCTTTC	f	4238	4260
P2x2r	5′ ACCGGATTCCAGCTGGGAAATG	r	1384	1405
P52	5′ CATTCCTCCTGGGACGC	r	2749	2765
P2558	5′ TGCTGAGGCTCCCCTACCAGA	f	301	321
P1	5′ TTACCCGCATCTCCCACCCCCAG	f	1912	1934
P4	5′ TTACCCGCATCTCCCACCCCCAA	f	1912	1934

NOTE.—Underlined nucleotides refer to mismatch bases. f = forward primer; r = reverse primer.
[a] Position according to Kimura et al. (1989).

ABI 373A automated sequencer and compared with the published wild-type *CYP2D6* sequence (Kimura et al. 1989). To confirm new allelic variants, which were found in three samples, DNA was reextracted once from frozen blood and taken for another PCR and sequencing procedure.

Statistical Calculations

Significance of differences of log MR in the dextromethorphan phenotyped group ($n = 456$) was tested (*a*) between carriers of three, two, one, and zero functional alleles, (*b*) between observed combinations of UM (ultrarapid metabolizer), EM, IM, or PM alleles, and (*c*) between all observed allele combinations, by use of one-way analysis of variance (ANOVA) with the BMDP 7D program (Dixon et al. 1992). Carriers of two and three functional alleles and of different genotypes were compared in the debrisoquine-phenotyped, relatively small group. After logarithmic transformation, 95% confidence limits of the mean values of the MR were calculated as mean MR ± 1.96 multiplied by the SEM.

Results

Frequency of CYP2D6 *Mutations and Their Allelic Linkage*

Frequencies of the *CYP2D6* point mutation, deletion, and hybrid alleles are presented in table 3. All mutation sites considered to be characteristic and function determining for an allele group were measured. The most frequent alleles were the *CYP2D6* wild-type **1* (.364), the *2 allele (.324), and the PM allele **4* (.207). The two IM alleles **9* and **10* were found with frequencies of .018 and .015, respectively, whereas the PM allele frequencies were .020 (**3* and **5*), .009 (**6*), and .001 (**7, *15,* and **16*). Although other functionally inactive alleles (*CYP2D6*8, *11, *12, *13,* and **14*) had been described in comparable Caucasian populations, they were not detected in any subject of this study.

Evaluation of Different CYP2D6 *Gene-Duplication Alleles*

In most cases of *CYP2D6* gene duplication, a duplication of two *CYP2D6*2* alleles could be unambiguously

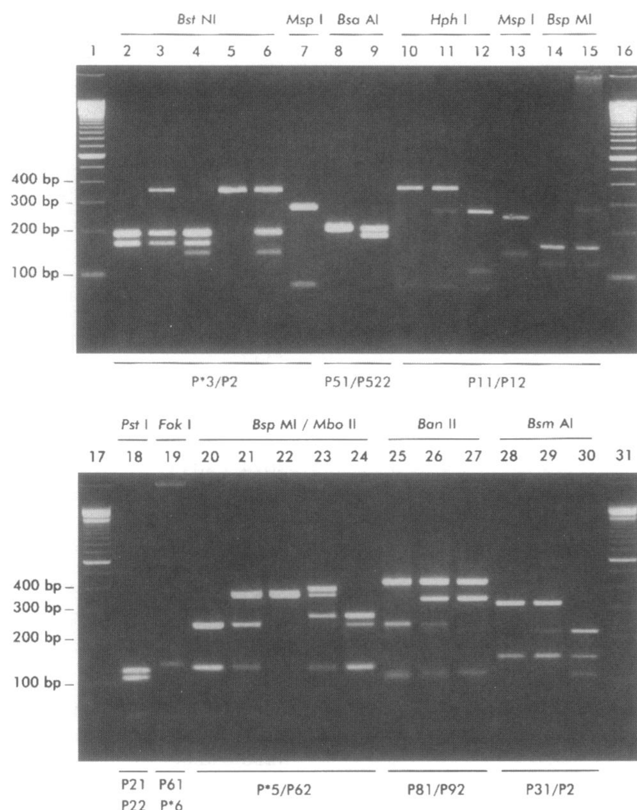

Figure 1 Restriction-band patterns of the point mutation–specific PCR-RFLP tests. Different allele constellations, derived from one PCR, are indicated by lines with respective PCR primers (as in table 1) on the bottom, and different alleles resulting from one enzyme digestion are indicated by lines and the respective enzymes on the top. *Upper panel,* 2 = G/G_{1934}, 3 = G/A_{1934}, 4 = $G_{1934}/DelT_{1795}$, 5 = A/A_{1934}, 6 = $A_{1934}/DelT_{1795}$, 7 = G/G_{1846}, 8 = A/A_{2637}, 9 = $A/DelA_{2637}$, 10 = C/C_{188}, 11 = C/T_{188}, 12 = T/T_{188}, 13 = G/G_{212}, 14 = G/G_{226}, and 15 = $G/InsT_{226}$. *Lower panel,* 18 = G/G_{971}, 19 = A/A_{3023}, 20 = C/C_{2938}, 21: C/T_{2938}, 22: T/T_{2938}, 23: T_{2938}/*9, 24: C_{2938}/*9, 25: G/G_{4268}, 26 = G/C_{4268}, 27 = C/C_{4268}, 28 = G/G_{1749}, 29 = G/C_{1749}, and 30 = C/C_{1749}. Lanes 1, 16, 17, and 31, 100-bp DNA molecular weight marker.

deduced from results of reactions 1–12. Further analysis was necessary, however, to distinguish between *2×2/*1 and *1×2/*2 genotypes and between *2×2/*4 and *4×2/*2 constellations. This was achieved by two PCR-RFLP tests for differentiation between duplicated *1, *2, or *4 alleles (described above) revealing six carriers of *1 duplications (allele frequency .005), one duplication of the defective *4 allele (.001), and 16 *2×2 carriers (.013). The *Xba*I RFLP analyses did not reveal fragments >44 kb. Therefore, we concluded that no higher *CYP2D6* amplifications than duplication were present in our sample.

Phenotype-Genotype Correlation

Observed *CYP2D6* genotypes first were classified into four groups of none, one, two, or three functional alleles. ANOVA revealed significant differences ($P < .001$) in metabolic activity between carriers of three (mean dextromethorphan MR = .002), two (.003), and one (.009), compared with carriers of no functional alleles (1.902), and between carriers of one versus two ($P < .001$) and three ($P < .01$) functional alleles. Second, when defective, intermediate, active, and ultrarapid alleles were taken into account, genotypes were classified into nine combinations as described in table 4. The functional in vivo role of the intermediate alleles (IM; *2, *9, and *10) was explored by comparison of the different combinations: significant differences ($P < .001$) were obtained not only between PM/PM and all other combinations but also between IM/PM and EM/EM, EM/IM, and IM/IM constellations; between EM/PM and EM/EM and EM/IM; and, with $P < .01$, between IM/PM and EM/PM constellations. When one compares the major IM allele *2 with the wild-type allele *1, there is a higher magnitude of mean dextromethorphan MR (fig. 2*A*) of *2/*2 compared with *1/*1 (not significant) and a significant difference ($P < .01$) between *1/*4 and *2/*4. In the smaller debrisoquine-tested group, the difference between *1/*4 and *2/*4 is significant, with $P < .05$ (fig. 2*B*).

In phenotyping, the antimode (cutoff point between EMs and PMs) was .3 for the dextromethorphan test (Schmid et al. 1985) and 12.6 for the debrisoquine test. The log MRs of dextromethorphan- and debrisoquine-tested subjects were each plotted against the observed *CYP2D6* allele combinations (fig. 2). Among the debrisoquine-tested individuals ($n = 133$), all eight PMs in phenotype were genotyped as PMs. In the dextromethorphan group, there was a high concordance between PM genotype and PM phenotype, with only one genotypically heterozygous PM (*2/*5 genotype) with a metabolic ratio of .47 (fig. 2*A*). This DNA sample was sequenced for validation as described above, but no further mutations were found, and the propositus was not available for a second phenotyping test. The MRs of two other subjects (.31 and .33) with *2/*4 genotype were, with regard to the variance of the HPLC procedure (coefficient of variance = 10%), insignificantly higher than .3. The MRs of these three samples were within the 95% confidence limits of their genotype, and all had combinations of one deficient allele, with one *2 allele corresponding to our observation of significantly reduced activity (fig. 2). In other words, all 41 PMs in phenotype found in this study were well explained by their genotype.

Novel CYP2D6 Allelic Variants

In one case of *2/*5 and unusually high MR (described above), sequencing showed no new mutations, and the genotype was confirmed. Three other samples were sequenced, since they had unexplained band patterns in PCR-RFLP tests, and this has revealed three novel variants of known *CYP2D6* alleles. The whole

Table 3

Frequency of *CYP2D6* Alleles (From a Sample of 589 Caucasian Individuals, i.e., 1,178 Alleles)

Allele	Alleles with Single-Base Changes at Nucleotide Number												Allele Frequency		
	188	212	226	971	1749[b]	1795	1846	1934	2637	2938	3023	4268	No.	%	95% CL (%)
*1[a]	C	G	G	G	G	T	G	G	A	C	A	G	429	**36.4**	33.7–39.2
*2	C	G	G	G	**C**	T	G	G	A	**T**	A	**C**	382	**32.4**	29.8–35.2
*3	C	G	G	G	G	T	G	G	**Del**	C	A	G	24	**2.04**	1.31–3.02
*4	**T**	G	G	G	**C/G**	T	G	**A**	A	C	A	**C**	244	**20.7**	18.4–23.1
*6	C	G	G	G	G	**Del**	G	G	A	C	A	G	11	**.93**	.47–1.66
*7	C	G	G	G	G	T	G	G	A	C	**C**	G	1	**.08**	.00–.47
*8	C	G	G	G	**C**	T	**T**	G	A	**T**	A	**C**	0	**.00**	.00–.31
*10	**T**	G	G	G	**C**	T	G	G	A	C	A	**C**	18	**1.53**	.91–2.40
*11	C	G	G	**C**	**C**	T	G	G	A	**T**	A	**C**	0	**.00**	.00–.31
*12	C	**A**	G	G	**C**	T	G	G	A	**T**	A	**C**	0	**.00**	.00–.31
*14	**T**	G	G	G	G	T	**A**	G	A	**T**	A	**C**	0	**.00**	.00–.31
*15	C	G	**Ins T**	G	G	T	G	G	A	C	A	G	1	**.08**	.00–.47
Data	Frequency of Point Mutations														
No.	262	0	1	0	641	11	0	244	24	382	1	644			
%	**22.2**	**.00**	**.08**	**.00**	**54.4**	**.93**	**.00**	**20.7**	**2.04**	**32.4**	**.08**	**54.7**			
Lower 95% CL (%)	19.9	.00	.00	.00	51.5	.47	.00	18.4	1.31	29.8	.00	51.8			
Upper 95% CL (%)	24.7	.31	.47	.31	57.3	1.66	.31	23.1	3.02	35.2	.47	57.5			
Alleles with major rearrangements:															
*1×2	(*CYP2D6*1* duplicated)												6	.51	.19 –3.33
*2×2	(*CYP2D6*2* duplicated)												16	1.34	.78 –2.20
*4×2	(*CYP2D6*4* duplicated)												1	.08	.00 .47
*5	(*CYP2D6* deleted)												23	1.95	1.24 –2.92
*9	(A_{2701}–A_{2703}) or (G_{2702}–A_{2704}) of *CYP2D6* deleted												21	1.78	1.11 –2.71
*13	(*CYP2D6/CYP2D7* hybrid, exon 1 *CYP2D7*, exons 2–9 *CYP2D6*)												0	.00	.00 .31
*16	(*CYP2D6/CYP2D7* hybrid, exon 1–7 *CYP2D7* related, exons 8–9 *CYP2D6*)												1	.08	.00 .47

[a] Wild type (wt).
[b] Although $G_{1749}C$ is a silent mutation in contrast to all other mutations mentioned here, it was included for validation of respective alleles due to its high frequency and occurence in several alleles.

CYP2D6 gene was sequenced in these cases, but no further associated mutations were found in addition to those described here.

One new variant of the wild-type *CYP2D6*1* allele (termed *CYP2D6*1C*) was disclosed in a subject originally found to have the **1/*1* genotype, having an extra band in gel electrophoresis of the *Bsm*AI-digested P31/P2 PCR product (reaction 9 in table 1), indicating the loss of one *Bsm*AI cleavage site (fig. 3). Sequencing revealed a T→C transition at nt 1957, which is a silent mutation ($G_{176}G$). The dextromethorphan MR (.002) of this person was not significantly different from those of 61 **1/*1* carriers.

Another *2 variant was found in a volunteer with the **2/*4* genotype. At nt 2558, a single C→T substitution was observed. To specify the mutation as being *2 or **4* related, from the whole *CYP2D6* PCR product, a nested amplification of only the *2 allele was achieved by use of primers P200 and P2558, which is specific for the **4* intron region between nt 302 and nt 333. Exons 2–9 were amplified from this PCR product and taken for sequencing, showing that the $T_{2558}C$ transition was on the *2 allele. To confirm this, a PCR-RFLP test was developed: from the P100/P200 PCR product, a 309-bp fragment was amplified with primers P51 and P52 (primers are listed in table 1; reaction conditions are as described above for the nested PCRs), and digestion with restriction enzyme *Fok*I gave an additional restriction site (fig. 3). Since the $T_{2558}C$ mutation is a "silent" mutation ($H_{232}H$), no functional consequences are expected. It is interesting that two of the eight **2B* carriers, who were also carriers of the defective **4* allele, showed dextromethorphan MRs near the antimode of .3 (MR_{II182} = .245, MR_{N173} = .330). This was significantly higher (ANOVA, $P < .05$) compared with 64 subjects with the **4/*2* constellation. Three other subjects had the **1/*

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Table 4

***CYP2D6* Allelic Groups Observed in 456 Dextromethorphan-Tested Caucasians**

	Frequency		Enzymatic Activity
CYP2D6 Genotype	No.	Percent (95% CL)	Mean of MR (95% CL of Mean MR)
UM/EM	3	.66 (.14–1.91)	.002 (.001–.003)
UM/IM	9	1.97 (.91–3.71)	.002 (.001–.006)
Σ3 active genes	12	2.63 (1.37–4.55)	.002 (.001–.005)[*]
EM/EM	62	13.6 (10.6–17.1)	.002 (.002–.003)[*,†,‡,§]
EM/IM	130	28.5 (24.4–32.9)	.003 (.002–.003)[*†,‡]
IM/IM	50	11.0 (8.25–14.2)	.004 (.003–.006)[*,†]
UM/PM	4	.24 (.36–2.23)	.005 (.002–.014)
Σ2 active genes	246	53.9 (49.2–58.6)	.003 (.002–.003)[*]
EM/PM	75	16.4 (13.2–20.2)	.006 (.004–.008)[*,‖]
IM/PM	90	19.7 (16.2–23.7)	.013 (.009–.020)[*]
Σ1 active gene	165	36.2 (31.8–40.8)	.009 (.007–.012)[*,#]
PM/PM	33	7.24 (5.03–10.0)	1.902 (1.392–2.598)

Note.—Statistical comparison was performed between means of MR of dextromethorphan in the different genotype groups (provided $n \geq 10$).

[*] $P < .001$ versus PM/PM

[†] $P < .001$ versus IM/PM

[‡] $P < .001$ versus EM/PM

[§] $P < .1$ versus IM/IM

[‖] $P < .01$ versus IM/PM

[#] $P < .001$ versus (Σ2 active genes); $P < .01$ versus (Σ3 active genes)

2B genotype, and the three remaining cases had the **2/*2B* genotype. No significant differences were found between MRs of the 3 **1/*2B* subjects and a group of 113 **1/*2* cases or between 2 dextromethorphan-tested **2/*2B* subjects and 37 **2/*2* cases.

A new rare variant of the **4* allele was discovered in a subject with the **1/*4* genotype, who carried mutations $C_{188}T$, $G_{1749}C$, $G_{1934}A$, $C_{2938}T$, and $G_{4268}C$, but the $C_{2938}T$ mutation was not previously described in **4* alleles. For corroboration that the $C_{2938}T$ mutation is **4* and not **1* associated, allele-specific PCR with **1*- and **4*-specific forward primers P1 or P4 and reverse primer P200 (described in table 1) was performed, followed by sequencing. The results confirm that $C_{2938}T$ is associated with *CYP2D6*4*. We suggest to term the novel allelic variants **1C, *2B,* and **4E,* respectively (table 5).

Discussion

Frequency of Poor Metabolizer Alleles

Population frequencies of known allelic variants of the highly polymorphic enzyme CYP2D6 have been determined in 589 unrelated volunteers of Caucasian (German) origin. Since the discovery of the *CYP2D6* polymorphism (Mahgoub et al. 1977; Eichelbaum et al. 1979), the poor metabolizers were the subgroup attracting greatest interest. In treatment with many drugs, substantially lower doses would be optimal for this subgroup; some other drugs that require CYP2D6 bioactivation, like codeine, should not be given. Optimally, all 12 disabling mutant alleles have to be determined for this purpose (fig. 4), but each additional PCR reaction increases also the costs. In our sample, the PM group (7.0%) was explained by the five PM alleles **3, *4, *5, *6,* and **15.* They were identified by reactions 1–4 (table 1), detecting alleles **3, *4, *5, *6, *8, *12, *14,* and **15.* Although the **5*-detecting assay seems to be unnecessary for PM identification—occurring heterozygous, the other PM allele will be detected homozygous by PCR-RFLP; occurring homozygous, there will be no *CYP2D6* amplification—the **5*-detecting PCR serves in the second case to prove the gene deletion and to avoid misclassification of poor DNA samples as **5/*5.* A screening using reactions 1–4 seems to be efficient and reliable enough to explain almost completely PM phenotype in comparable Caucasian populations.

CYP2D6 *Ultrarapid Metabolizer Alleles*

Among dextromethorphan-tested subjects, discrimination between extensive and ultrarapid metabolizers (three functional alleles) was not sufficient, because dextromethorphan was almost completely metabolized in ~3% of extensive metabolizers (concentration < 10 ng/ml). Since the turnover rate is much higher for dextromethorphan than for debrisoquine (Appanna et al. 1996), the increased enzyme amount caused by the gene

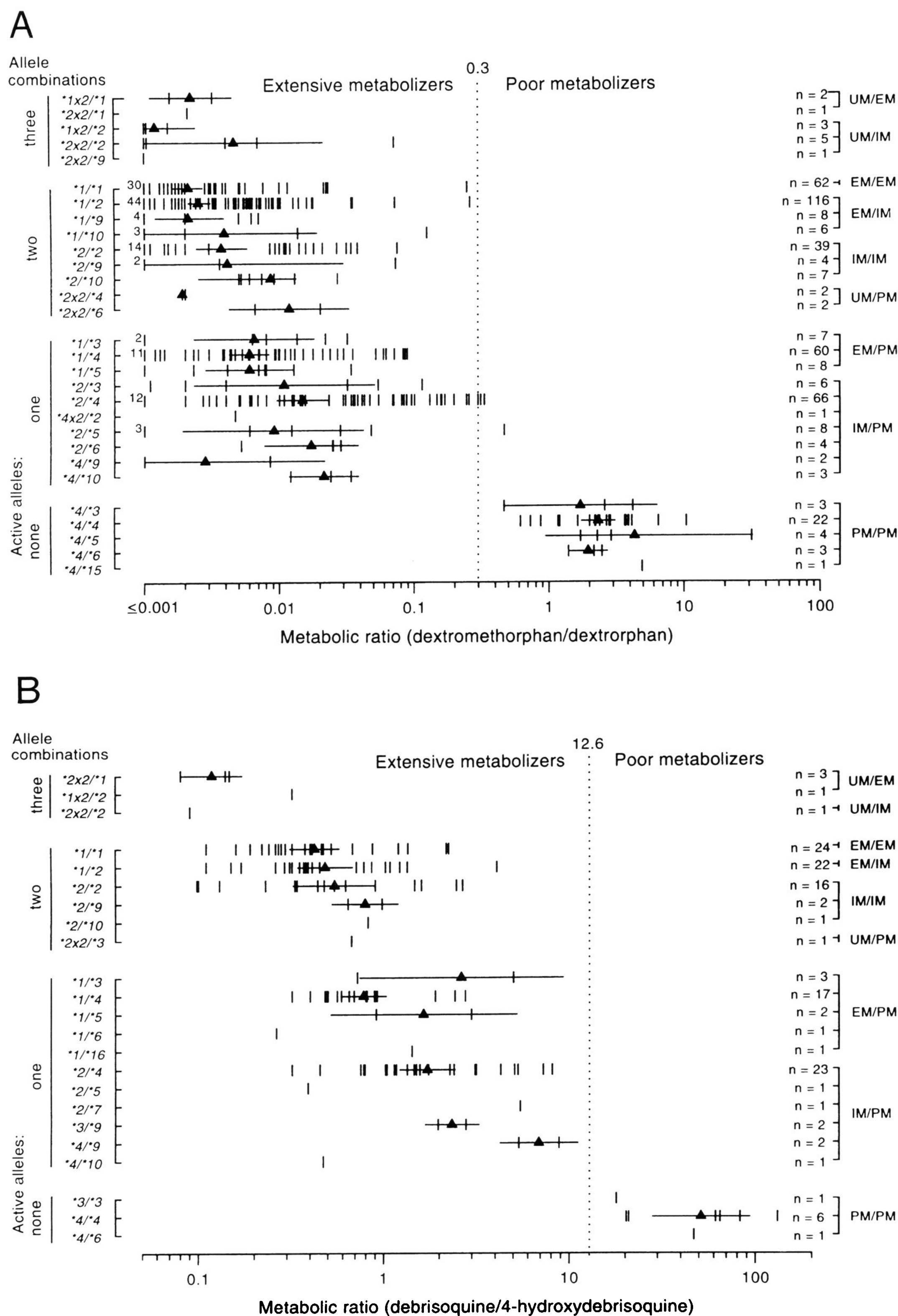

Figure 2 CYP2D6 phenotypes versus *CYP2D6* allele combinations from 589 Caucasians. Means of MR of the respective allelic combinations are indicated by triangles. Horizontal lines indicate the 95% confidence limits of mean MR. Statistical comparison between means of MR was performed in genotypes with $n \geq 10$. *A*, Metabolic ratios and *CYP2D6* genotypes of 456 dextromethorphan-tested subjects. Numbers of subjects with MRs $\leq .001$ are indicated. Significant differences were as follows: **4/*4* with $P < .001$ versus **1/*1*, **1/*2*, **2/*2*, or **1/*4*; **2/*4* with $P < .001$ versus **1/*1*, **1/*2*, **2/*2*, or **4/*4*; **1/*4* with $P < .001$ versus **1/*1* or **1/*2*; and **1/*4* with $P < .01$ versus **2/*4*. *B*, Metabolic ratios and *CYP2D6* genotypes of 133 debrisoquine-tested subjects. Significant differences were as follows: **2/*4* with $P < .001$ versus **1/*1*, **1/*2*, or **2/*2*; and **2/*4* with $P < .05$ versus **1/*4*.

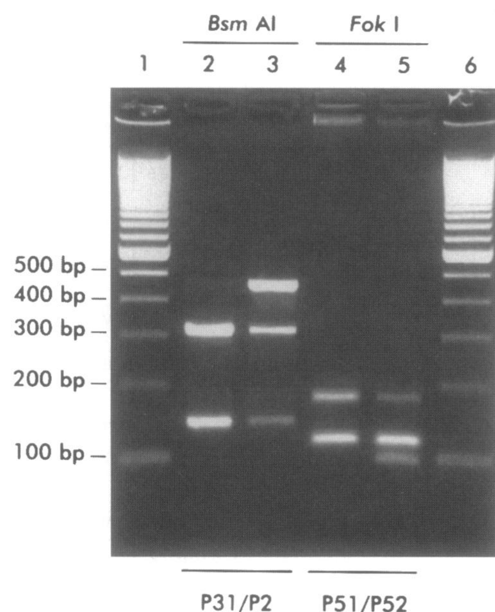

Figure 3 Electrophoretic patterns of the novel point mutations $T_{1957}C$ (*1C)– and $T_{2558}C$ (*2B)–detecting PCR-RFLP tests. Lanes 1 and 6, 100-bp molecular weight marker. Lane 2, *Bsm*AI digest (311/156 bp) of the P31/P2 PCR product of a wild-type T/T_{1957} carrier. Lane 3, heterozygous T/C_{1957} carrier (467/311/156 bp). Lane 4, *Fok*I digest of the P51/P52 PCR product of a wild-type T/T_{2558} sample (172/23/114 bp). Lane 5, heterozygous T/C_{2558} subject (172/88/84/23/114 bp).

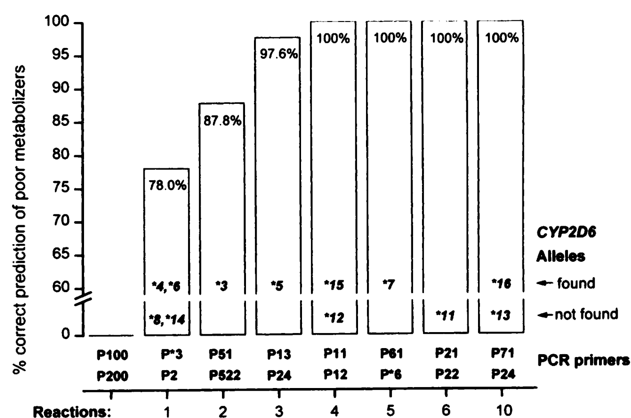

Figure 4 Correctness of prediction of PM phenotype of CYP2D6 among 589 Caucasians of this study (dextromethorphan- and debrisoquine-tested groups), with increasing numbers of PCR methods used for detection of specific PM alleles (see table 1; reactions 7–9 for detection of IM alleles are not included).

duplication becomes apparent more easily with debrisoquine testing (Dahl et al. 1995); in the debrisoquine-tested group, carriers of three and two functional alleles differed significantly in their metabolic capacity ($P < .01$, *t*-test). Despite of this advantage in finding UMs, for legal reasons, debrisoquine, which is not approved as a drug in many countries, was frequently replaced by dextromethorphan during the past 10 years (Brockmöller and Roots 1994).

The *CYP2D6* gene duplication was described to be heterogenous: there are **1* duplications (Dahl et al. 1995) as well as cases of a **4* duplication (in Africans and Black Americans [Masimirembwa et al. 1993; Løvlie et al. 1996]) in addition to the known *2 duplication. Although **4* duplications are not yet described among Caucasians, all three kinds of allele duplications were found in our sample. Whereas differences in metabolic capacity between *1×2 and *2×2 carriers should be very slight, without importance for clinical routine, misclassification of cases with **4* duplications as ultrarapid metabolizers can be dangerous in a routine prediction of a patient's metabolic capacity, as discussed by Løvlie et al. (1996). The additional nested PCR assay from the 10-kb product as presented above seems to be a good routine method to avoid such misclassification.

Table 5

Characteristics of the New Allelic Variants and their Frequencies among Caucasian Individuals

CYP2D6 Allele Name	Nucleotide Changes	Effect on the Protein	Allele Frequency: No.	Allele Frequency: Percent	Allele Frequency: 95% CL
*1C	$T_{1957}C$	$G_{176}G$	1	% of all[a]: .08	.00–.47
				% of *1[b]: .23	.01–1.29
*2B	$G_{1749}C$, $T_{2558}C$, $C_{2938}T$, $G_{4268}C$	$V_{136}V$, $H_{232}H$, $R_{296}C$, $S_{486}T$	8	% of all[a]: .68	.29–1.33
				% of *2[c]: 2.09	.91–4.08
*4E	$C_{188}T$, $G_{1749}C$, $G_{1934}A$, $C_{2938}T$, $G_{4268}C$	Splicing defect	1	% of all[a]: .08	.00–.47
				% of *4[d]: .41	.01–2.26

[a] n = 1,178 alleles (589 individuals).
[b] n = 429 alleles.
[c] n = 382 alleles.
[d] n = 244 alleles.

Role of Intermediate Active Alleles

The IM alleles (*2, *9, *10) in a homozygous manner (e.g., *2/*2) or together with defective alleles (e.g., *2/*4) were, as expected, not associated with the PM phenotype, but, when combined with PM alleles (IM/PM, mean dextromethorphan MR = .014), they showed a significantly higher metabolic ratio than did the EM/PM (mean MR = .006) genotype (table 3). Although the *2 allele was reported to have catalytic activity similar to that of *1 (Johansson et al. 1993), we found significant differences between *1/*4 and *2/*4 in both the dextromethorphan- and debrisoquine-tested groups (fig. 2). Although the magnitude of this effect is relatively small, it indicates a significantly decreased activity of *2 compared with *1. Therefore, defining "intermediate" as significantly lower than wild-type, we considered *2 as an IM allele. Determination of IM alleles, especially of *2, for clinical purposes may be unnecessary, but they may explain sources of interindividual variability in drug trials.

Heterogeneity in CYP2D6 *Allele Frequencies within Caucasians*

Ten different alleles have been detected here, but six other alleles described earlier in comparable populations were not found. Frequencies of the defective alleles *3, *4, *5, and *6 found in our study are compatible with earlier data (Broly et al. 1991; Daly et al. 1991; Evert et al. 1994*a;* Saxena et al. 1994; Tefre et al. 1994). In these studies, a proportion of 86%–100% of PMs was explained by these four alleles; in our study, 100%, including one additional case with the rare *15 allele, was. Minor differences within different Caucasian populations, depending on the ethnic origin, cannot be ruled out, especially for the rare alleles. The PM alleles *8, *11, *12, *13, and *14 (some of them are rare even in the populations in which they were originally described) were not observed here, although all samples were analyzed for them. The *7 allele, which was discovered among 100 Caucasians with 3 cases (Evert et al. 1994*b*), was detected with 1 case among 589 subjects in our study, a discrepancy which is significant (P = .0005, Fisher's exact test). This discrepancy may be the consequence of historical mixing of the southern German population with Mediterranean populations. The carrier of a *CYP2D6*15* allele in this study is identical with the volunteer in whom the allele was originally found (Sachse et al. 1996).

The frequencies of the gene-deletion and the gene-duplication alleles seem to differ between ethnic groups, when our data are compared with the results of Agúndez et al. (1995). Among 217 Spaniards, the frequency of the gene deletion *CYP2D6*5* was .039, compared with .020 in our sample, and the proportion of the gene-duplication alleles was .035, compared with .020 in our population. This may partly be explained by historical immigration of populations from North Africa in Spain, which are known to have *CYP2D6* gene duplication and multiduplications with an allele frequency ≤.16 (in Ethiopians; Aklillu et al. 1996).

Three new variants of known *CYP2D6* alleles were detected, which indicates the considerable variability of the CYP2D6 gene locus. Such mutations may explain allele evolution or ethnic origin of alleles, as pointed out by Daly et al. (1996*a*).

Methodological Aspects

Point mutations in this study were exclusively tested by RFLP analyses after PCR. Although the first described methods for *CYP2D6* genotyping based on allele-specific PCR (Heim and Meyer 1990), investigators changed to PCR-RFLP techniques (Gough et al. 1990; Tefre et al. 1994). By use of mismatch primers, all point mutations known in *CYP2D6* could be detected by PCR-RFLP analysis, which is, in our opinion, of higher reliability compared with allele-specific PCR with different 3′ nucleotides complementary to the respective alleles.

Clinical Applications

In clinical pharmacology, CYP2D6 genotyping was awaited with great expectations, since individual differences in drug disposition could be compensated for in part by dose adjustment according to the genetically predicted metabolic phenotype (Brockmöller and Roots 1994). Although this is feasible now, and specificity and sensitivity of PCR-based prediction of CYP2D6 activity is better than in many other routinely used medical laboratory or radiological tests, genotyping of *CYP2D6* is still not routinely used in clinical medicine. One reason may be that the description of so many mutations has produced doubts about what is to be determined routinely. Our data, derived from a relatively large sample, should help in this situation: we recommend performing PCR reactions 1–4 of table 1 routinely, to detect the most frequent PM alleles, and further recommend a test for the *CYP2D6* gene duplication (reaction 12). We presented an additional test to avoid misclassification of *4 duplications as ultrarapid alleles (reaction 12B, recommended in cases with a gene duplication and a *4 allele). Still, the value of genotyping in those medical specialties where CYP2D6-metabolized drugs are frequently applied (e.g. neuroleptics, antidepressants, beta-blockers, antiarrythmics, or codeine) will have to be evaluated.

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