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(54) Title: DETECTION OF CYP1A1, CYP3A4, CYP2D6 AND NAT2 VARIANTS AND THERAPEUTIC USES THEREOF		
(57) Abstract		
<p>The present invention relates to the detection of genetic polymorphisms in gene encoding xenobiotics metabolizing enzymes CYP1A1, CYP2D6, CYP3A4, and NAT2. In particular, the present invention relates to a method of detecting genetic variation in individuals by amplifying the locus of interest, hybridizing the resulting amplification product with allele-specific-oligonucleotides (ASOs), and visualizing the results. Such a method is in the field of diagnostics, pharmacogenetics, therapeutics, and drug metabolism.</p>		

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DETECTION OF CYP1A1, CYP3A4, CYP2D6 AND NAT2
VARIANTS AND THERAPEUTIC USES THEREOF

BACKGROUND OF THE INVENTION

(a) Field of the Invention

5 This invention relates to the detection of genetic polymorphisms in gene encoding xenobiotics metabolizing enzymes CYP1A1, CYP2D6, CYP3A4, and NAT2. In particular, the present invention relates to a method of detecting genetic variation in individuals by
10 amplifying the locus of interest, hybridizing the resulting amplification product with allele-specific-oligonucleotides (ASOs), and visualizing the results. Such a method is in the field of diagnostics, pharmacogenetics, therapeutics, and drug metabolism.

15 (b) Description of Prior Art

 It is recognized that marked interindividual differences exist in enzymes that metabolize xenobiotics and that this variability can be genetically determined. Considerable progress has been made in
20 identifying a number of individual enzymes that are subject to genetic polymorphism. A polymorphism is generally considered to be a stably inherited trait. Genetic variation means differences between individuals in regard to the base sequence of their DNA. Genotyping
25 involves identification of (defined) genetic mutations that, in a particular case, give rise to the specific drug metabolism phenotypes. These mutations include genetic alterations that lead to increased activity, absence of an active protein (null allele) or
30 production of a mutant protein with altered catalytic capacity. A number of types of genetic polymorphism in xenobiotics metabolizing enzymes are relevant to clinical practice. These include those relating to N-acetyltransferase 2 (NAT2) and the cytochromes P450 1A1
35 (CYP1A1), 3A4 (CYP3A4) and 2D6 (CYP2D6). Genetic

variation can be detected by means of electrophoresis of DNA fragments, generated by digestion of PCR products with a restriction enzyme the so-called PCR-RFLP approach. This method that has proven useful in
5 screening for genetic mutations associated with altered metabolism of drugs and/or cancer susceptibility. It consists of amplification of a specific region of the gene of interest by PCR followed by digestion of the amplified DNA product with restriction endonucleases.
10 Restriction endonucleases have the capacity to digest DNA with a high degree of nucleotide sequence specificity. Thus, point mutations within the recognition sequence of a specific restriction endonuclease may be detected through determining whether the DNA of
15 interest serves as a substrate for that endonuclease. These studies are routinely carried out by comparing the size of digestion products generated from a DNA substrate amplified from control subject DNA with respect to study subject DNAs. The size of the diges-
20 tion products is easily evaluated by agarose gel electrophoresis with ethidium bromide staining and UV transillumination. In this case the position of the bands varies between individuals, as a result of gain or loss of the recognition site for the restriction
25 enzyme used, this is restriction fragment length polymorphism (RFLP). However, the PCR-RFLP is not well suited for genotyping a considerable number of samples that are usually analyzed in such investigations.

It would be highly desirable to be provided with
30 a method to detect genetic polymorphisms in gene encoding xenobiotics metabolizing enzymes CYP1A1, CYP2D6, CYP3A4, and NAT2.

It would be highly desirable to be provided with
an assay to detect functional polymorphisms (variants
35 or mutations) in CYP1A1, CYP2D6, CYP3A4 and NAT2 genes.

SUMMARY OF THE INVENTION

The present invention relates to the development of an assay to detect functional polymorphisms (variants or mutations) in CYP1A1, CYP2D6, CYP3A4 and
5 NAT2 genes.

The presence of these mutant alleles is associated with an altered enzyme activity potentially leading (i) to toxicity when individuals are treated with standard doses of certain prescribed drugs; (ii)
10 to increased susceptibility to cancer following environmental exposures; or (iii) other clinical condition. Detection of DNA variants at the CYP1A1, CYP2D6, CYP3A4 and NAT2 loci offers a strategy for identifying individuals «at risk» based on their
15 genotype, prior to treatment with potentially toxic doses of drugs or to exposure to environmental toxins.

In accordance with the present invention, the detection of genetic polymorphisms in gene encoding xenobiotics metabolizing enzymes CYP1A1, CYP2D6,
20 CYP3A4, and NAT2 or any other functional polymorphisms (variants or mutations) may be effected using any known state-of-the-art hybridization approaches, including, but not limited to, Southern blot, reverse dot-blot and liquid phase hybridization.

25 Reverse dot blot refers to a treatment of a support (such as nylon membrane) to which is attached an ASO capable of hybridizing with a labeled complementary probe (such as amplified DNA).

In accordance with another embodiment of the
30 present invention, the detection of specific mutations within a gene of interest is through hybridization of PCR products with allele-specific oligonucleotide (ASO) probes for the wild type or variant alleles utilized in parallel hybridizations. Only the oligonucleotide that
35 precisely hybridizes to the target sequences produces a

signal from a labeled probe. This genotyping method, which require small amounts of nucleated cells derived from a variety of sources, is not affected by the underlying disease or by drugs taken by the patient.
5 It provides results within 24-48h, allowing for rapid intervention.

One aim of the present invention is to provide a diagnostic test to identify individuals with altered xenobiotics-metabolizing activities based on their
10 genotypes. Such diagnostic test to determine genotype of individuals is quite advantageous because measuring the enzymatic activity has many limitations. To achieve this goal, different tests have been developed to detect mutations in the genes CYP1A1, CYP2D6, CYP3A4
15 and NAT2. These tests involved amplification of these genes where the mutations of interest are found. Following amplification, the amplified fragments are assayed for the presence or absence of the specific mutation of interest (i.e. at least one of the
20 mutations) by using hybridization with ASO probes. Although much of these assays can be done in any molecular biology facilities, procedures and kits are designed that contain all the reagents, primers and solutions for the genotyping test to facilitate the
25 procedure for use in general clinical laboratories, such as those found in a typical hospital, clinic and even private reference laboratories.

In accordance with the present invention there is provided an isolated oligonucleotide molecule
30 comprising a sequence hybridizing to a gene encoding xenobiotics metabolizing enzymes CYP1A1, CYP2D6, CYP3A4, or NAT2, wild type and mutant alleles thereof; wherein said sequence is sufficiently complementary to said gene to hybridize therewith.

In accordance with the present invention there is provided an isolated oligonucleotide molecule comprising a mutant allele of CYP1A1, which contains a point mutation at one position selected from the group consisting of position 4887, 4889, 6235 and 5639, such as an adenine at position 4887, a guanine at position 4889, a cytosine at position 6235 and a cytosine at position 5639. Preferred mutant oligonucleotide molecules have a nucleic acid sequence as set forth in SEQ ID NOS:27, 28 and 31.

In accordance with the present invention there is provided an isolated oligonucleotide molecule comprising a wild-type allele of CYP1A1, which contains a normal nucleotide at one position selected from the group consisting of position 4887, 4889, 6235 and 5639, such as a cytosine at position 4887, a adenine at position 4889, a cytosine at position 6235 and a cytosine at position 5639. Preferred wild type oligonucleotide molecules have a nucleic acid sequence as set forth in SEQ ID NOS:26, 29 and 30.

In accordance with the present invention there is provided an isolated oligonucleotide molecule comprising a mutant allele of CYP3A4, which contains a point mutation at position -290, such as a guanine. Preferred mutant oligonucleotide molecule has a nucleic acid sequence as set forth in SEQ ID NO:37.

In accordance with the present invention there is provided an isolated oligonucleotide molecule comprising a wild-type allele of CYP3A4, which contains a normal nucleotide at position 290, such as an adenine. Preferred wild type oligonucleotide molecule have a nucleic acid sequence as set forth in SEQ ID NO:36.

In accordance with the present invention there is provided an isolated oligonucleotide molecule comprising a mutant allele of CYP2D6, which contains a

point mutation at one position selected from the group consisting of position 1934, 2637, 188, 212, 226, 971, 1111, 1726, 1795, 1846, 2064, 2701-2703, 2702-2704, and 3023, such as an adenine at position 1934 and a
5 deletion at position 2637, a thymine at position 188, an adenine at position 212, an insertion of a thymine at position 226, a cytosine at position 971, a thymine at position 1111, a cytosine at position 1726, a deletion of a thymine at position 1795, a thymine at
10 position 1846, an adenine at position 1846, an adenine at position 2064, a three-nucleotide deletion (adenine-guanine-adenine) at positions 2701-to-2703, a three-nucleotide deletion (guanine-adenine-guanine) at positions 2702-to-2704, and a cytosine at position
15 3023. Preferred mutant oligonucleotide molecules have a nucleic acid sequence as set forth in SEQ ID NOS:33 and 35.

In accordance with the present invention there is provided an isolated oligonucleotide molecule comprising a wild-type allele of CYP2D6, which contains a
20 normal nucleotide at one position selected from the group consisting of position 1934, 2637, 188, 212, 226, 971, 1111, 1726, 1795, 1846, 2064, 2701-2703, 2702-2704, and 3023, such as a guanine at position 1934, an adenine at position 2637, a cytosine at position 188, a cytosine at position 212, no insertion at position 226, a guanine at position 971, a cytosine at position 1111, a guanine at position 1726, a thymine at position 1795, a guanine at position 1846, a guanine at position 2064,
25 an adenine-guanine-adenine at positions 2701-to-2703, a guanine-adenine-guanine at positions 2702-2704, and an adenine at position 3023. Preferred wild type oligonucleotide molecules have a nucleic acid sequence as set forth in SEQ ID NOS:32 and 34.
30

In accordance with the present invention there is provided an isolated oligonucleotide molecule comprising a mutant allele of NAT2, which is at least 11 consecutive bases long and contains a point mutation at one position selected from the group consisting of position 341, 481, 590, 803, 857, 191, 282, 434, and 845, such as a cytosine at position 341, a thymine at position 481, an adenine at position 590, a guanine at position 803, an adenine at position 857, an adenine at position 191, a thymine at position 282, a cytosine at position 434, and a cytosine at position 845. Preferred mutant oligonucleotide molecules have a nucleic acid sequence as set forth in SEQ ID NOS:16, 18, 20, 23, and 24.

In accordance with the present invention there is provided an isolated oligonucleotide molecule comprising a wild-type allele of NAT2, which contains a normal nucleotide at one position selected from the group consisting of position 341, 481, 590, 803, 857, 191, 282, 434, and 845, such as a thymine at position 341, a cytosine at position 481, a guanine at position 590, an adenine at position 803, a guanine at position 857, a guanine at position 191, a cytosine at position 282, an adenine at position 434, and an adenine at position 845. Preferred wild type oligonucleotide molecules have a nucleic acid sequence as set forth in SEQ ID NOS:17, 19, 21, 22, and 25.

In accordance with another embodiment of the present invention there is provided an oligonucleotide molecule complementary to any of the oligonucleotide molecules identified as SEQ ID NOS:16-37.

In accordance with the present invention there is provided a diagnostic assay for determining genetic variants in CYP1A1, CYP3A4, CYP2D6 or NAT2 gene in a subject, which comprises the steps of:

a) obtaining a genomic DNA sample of said subject;

b) using the DNA sample of step a), amplifying a fragment comprising a polymorphic site of at least one
5 of CYP1A1, CYP3A4, CYP2D6 and NAT2 genes;

c) hybridizing the amplified fragment of step b) with allele-specific oligonucleotides (ASO) probes corresponding to wild type and variant alleles to determine CYP1A1, CYP3A4, CYP2D6 or NAT2 genotype of said
10 subject.

In accordance with a preferred embodiment of the present invention, the amplifying step b) is effected with PCR primers set forth in Table 1.

In accordance with a preferred embodiment of the present invention, the method further comprises a step
15 i) before step c) consisting in subjecting the amplified fragment of step b) to Southern dot blot transfer on membrane, and wherein step c) is effected by hybridizing the dot blots with the oligonucleotide.

In accordance with a preferred embodiment of the present invention, a labeled ASO probe is used in step
20 c) and is selected from the sequences set forth in Table 2 and hybridizes under stringent conditions (Table 2).

In accordance with the present invention there is provided a diagnostic kit for determining DNA variants in CYP1A1, CYP3A4, CYP2D6 or NAT2 gene in a subject, which comprises:

a) at least one of the PCR primers set forth in SEQ
30 ID NOS:1-15; and

b) at least one of the ASO probes set forth in SEQ ID NOS:16-37.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates common variants in the CYP1A1 locus;

Fig. 2 illustrates common variants in the CYP2D6 locus;

Fig. 3 illustrates common variants in the CYP3A4 locus; and

Fig. 4 illustrates common variants in the NAT2 locus;

Figs. 5A to 5C illustrate representative results of the PCR-ASO hybridizations of mutations in the CYP1A1, CYP2D6, CYP3A4 and NAT2 loci.

DETAILED DESCRIPTION OF THE INVENTION

A large number of enzymes exist to metabolize xenobiotics including drugs and environmental pollutants. Most of them have been classified as belonging to phase I or phase II pathways of metabolism. Virtually all chemicals ingested or absorbed by the body will be metabolized to some degree. Phase I enzymes include reductases, oxidases and hydrolases, while the phase II enzymes are all transferases. These reactions serve to transform a hydrophobic compound into a form that is more water soluble and can be easily eliminated from the organism through urine or bile. Polymorphism in xenobiotics metabolizing enzymes can be associated with marked differences in response to drug therapy and/or may also cause increased susceptibility to environmentally based diseases such as cancer. Differences in metabolism of therapeutics can lead to severe toxicity or therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically active drug. The variability in metabolizing capacity is due to the presence of mutant alleles for which the differences in DNA sequence from

the wild-type allele have been established, the prerequisite for the development of a genetic test for genotyping. Thus, in pharmacogenetic studies one applies genotyping of polymorphic alleles encoding drug-metabolizing enzymes to the identification of an individual's drug metabolism phenotype. A unique combination of polymorphism in 2 or more of these enzymes might render an individual susceptible to diseases such as cancer or neurotoxicity. Determination of these genetic polymorphism may be of clinical value in predicting adverse or inadequate response to certain therapeutic agents and in predicting increased risk of environmental or occupational exposure-linked disease.

Individuals that possess modified abilities to metabolize carcinogens are at increased risk of cancer. This is because sporadic cancers result from mutations in transforming genes and carcinogen-detoxification influences the mutational events in these genes. Polymorphisms in genes encoding carcinogen-metabolizing enzymes may have relevance in determining susceptibility to cancer individuals carrying the more active form of an enzyme involved in the activation of carcinogens or less efficient alleles of detoxifying enzymes, will be at greater risk of cancer. For this reason, two classes of genes have attracted interest: cytochromes P-450 and N-acetyltransferases. Genetic variants have been described in CYP1A1, CYP2D6 and NAT2 genes. Several studies have reported associations between these variants and altered risk of a variety of cancers, including those of the lung, bladder, gastrointestinal tract, skin, cervix and breast, as well as variability in drugs metabolism. The prevalence of each polymorphism varies greatly among different ethnic groups, as well as within the Caucasian population.

There is a growing interest in analyzing these loci, in the context of diverse molecular epidemiology studies. To date the mainstay of the NAT and CYP mutations genotyping has been PCR amplification of the DNA region of interest, followed by RFLP analysis of each known mutation (Cascorbi I et al. 1996a, *Cancer Res* 56:3961-3966; Cascorbi I, Brockmöller J, Roots, 1996b, *Cancer Res* 56:4965-4969; Sachse C et al., 1997, *Am J Hum Genet* 60:28; Shields PG et al., 1993, *Cancer Res.* 53:3486-3492; Taioli E et al., 1995, *Toxicol Let* 77:357 Sachse et al. 1997, Shields et al 1993, Taioli et al. 1995). To increase the efficiency and facilitate processing of the large number of samples we developed a combined approach including PCR dot blot and allele-specific oligonucleotide (ASO) hybridization. The assays involve pairs of PCR primers to amplify the common allelic variants CYP1A1*2A, *2B and *4, CYP2D6*3 and *4, CYP3A4*2, as well as NAT2*5A, *5B, *5C, *6A, and *7B (illustrated in Figs. 1-4). Based on the sequence of the mutant alleles provided herein, PCR primers are constructed that are complementary to the region flanking the point mutations. A primer consists of a consecutive sequence of nucleotides complementary to any region in the allele flanking the position, which is mutated in the mutant allele. According to the method of the present invention, once an amplified fragment is obtained, we can determine whether the individual tested is homozygous or heterozygous for a given DNA variant by hybridizing the dot blotted PCR fragments with ASO probes for the mutant or the wild-type alleles in parallel.

This approach has a potential for automation through the use of the 96-well microplates and robotic workstations for high sample throughput. PCR-ASO hybridization assay appears simple, efficient and cost

effective, particularly if a large number of samples are to be screened for several DNA variants.

N-acetyltransferase

N-acetyltransferase 2 (NAT2) is among the earliest discovered genetic polymorphism in xenobiotics-metabolizing enzymes. Deficiency in NAT2 is found in up to 50% of Caucasians and is responsible for toxicity associated with several drugs and susceptibility to certain types of cancer. The differences in the metabolism of these compounds distinguished phenotypically slow and fast acetylators. The slow acetylation phenotype frequency worldwide ranges from about 0.1 in the Japanese population to more than 0.9 in some Mediterranean peoples. Several mutant NAT2 alleles have been found in Caucasians and Asian individuals and these can be determined by PCR-RFLP genotyping (Blum et al. 1991; Hickman and Sim 1991; Rothman et al. 1993). Five mutations T341C, 481T, G590A, A803G and G857A account for nearly all slow acetylator alleles. Other known functional variants include: G191A, C282T, A434C and A845C.

Cytochromes P450

The major route of phase I drug metabolism is oxidation by cytochrome P-450 (CYP). Most clinically used drugs are metabolized to some degree by P450s. These enzymes are also principally responsible for activation of procarcinogens and promutagens.

CYP2D6. Debrisoquine 4-hydroxylase (CYP2D6) is the most well characterized P450 polymorphism. About 25% of prescribed drugs are metabolized by CYP2D6. The polymorphism appears to have clinical consequences in the use of cardiovascular drugs and drugs used for treatment of psychiatric disorders. Genotype has been shown to closely correlate with phenotypes. It has been argued that determination of a patient's CYP2D6

genotype should be a prerequisite for treatment with antipsychotic drugs. Several null CYP2D6 alleles have been characterized and PCR-RFLP assays have been developed for convenient genotyping (Gonzalez and Meyer 5 1991). The most common alleles are CYP2D6*3 (1bp deletion at pos. A2637) and *4 (splice-site mutation G1934A), accounting for over 96% of all null alleles. Individuals homozygous for any of these null alleles, completely lacking CYP2D6 activity, will be considered 10 phenotypically poor metabolizers (PM). There are several other less common polymorphisms: C188T, C212A, insT226, G971C, C1111T, G1726C, delT1795, G1846T, G1846A, G2064A, delA2701-A2703, delG2702-G2704, and A3023C. There are significant interethnic differences 15 in the prevalence of the PM phenotype of CYP2D6. For example, in North American and European Caucasian populations, the prevalence of poor metabolisers is 5-10%. In contrast, the prevalence is 1.8% in American blacks, 1.0% in Chinese, and apparently absent in the 20 Japanese population.

CYP1A1. Aryl hydrocarbon hydroxylase (CYP1A1) catalyses the first step in the metabolism of polycyclic aromatic hydrocarbons to carcinogens. Three CYP1A1 polymorphisms have been identified in Caucasians: transition T-to-C at position 6235 (T6235C) in the 3'-flanking region, substitution A-to-G in exon 7 (G4889A) exchanging the isoleucine 462 by valine in the heme binding region, and a C-to-A transversion at position 4887 (C4887A) substituting threonine to asparagine in 30 codon 461. The frequency of the three polymorphisms shows racial differences. Another variant, T5639C, is more prevalent in African-Americans.

CYP3A4. CYP3A4 is involved in the metabolism of numerous human carcinogens, steroid hormones, and 35 drugs. Until the present invention, no genetic variant

had been reported that explains the interindividual variability in CYP3A4 enzyme activity. We have identified a variant located in the 5'-untranslated region of the CYP3A4 gene. The frequency of this variant allele was estimated to be 2% in a Caucasian Canadian control population. The phenotypic consequence of this mutation is unknown but its presence within the transcriptional regulatory element NFSE suggests a potential role as functional variant.

10 **Mutations of CYP1A1, CYP2D6, CYP3A4 and NAT2**

In particular, the present invention relates to the design and utilization of oligonucleotide molecules comprising a mutant allele of CYP1A1 which contains a point mutation in at least one of the positions 4887, 4889, or 6235. The point mutation at 4887 is adenine and the whole oligonucleotide has the sequences shown in Table 2. The point mutation at 4889 is guanine and the whole oligonucleotide has the sequences shown in Table 2. The point mutation at 6235 is cytosine and the whole oligonucleotide has the sequences shown in Table 2. The invention relates to the design and utilization of oligonucleotide molecules comprising a wild-type (no mutation) allele of CYP1A1 which contains the normal nucleotide in at least one of the positions 4887, 4889, or 6235. The normal residue at 4887 is cytosine and the whole oligonucleotide has the sequences shown in Table 2. The normal residue at 4889 is adenine and the whole oligonucleotide has the sequences shown in Table 2. The normal residue at 6235 is thymine and the whole oligonucleotide has the sequences shown in Table 2.

Table 1

PCR primers for amplification of CYP1A1, CYP2D6, CYP3A4
and NAT2 mutant alleles

Locus	Mutation	PCR primer	SEQ ID NO:	Product Size
NAT2	T341C	P100, GTCACACGAGGAAATCAAATGC	SEQ ID NO:1	442 bp
		R1, ACCCAGCATCGACAATGTAATTCCTGCCCTCA	SEQ ID NO:2	
	C481T	P100, GTCACACGAGGAAATCAAATGC	SEQ ID NO:1	1211 bp
		P56, GTTTTCTAGCATGAATCACTCTGC	SEQ ID NO:3	
	G590A	P87, CCTGGACCAAATCAGGAGAG	SEQ ID NO:4	421 bp
P90, ACACAAGGGTTTATTTTGTTC		SEQ ID NO:5		
A803G	P87, CCTGGACCAAATCAGGAGAG	SEQ ID NO:4	421 bp	
	P90, ACACAAGGGTTTATTTTGTTC	SEQ ID NO:5		
G857A	P100, GTCACACGAGGAAATCAAATGC	SEQ ID NO:1	1211 bp	
	P56, GTTTTCTAGCATGAATCACTCTGC	SEQ ID NO:3		
CYP1A1	T6235C	M3F, GGCTGAGCAATCTGACCCTA	SEQ ID NO:6	899 bp
		P80, TAGGAGTCTTGTCTCATGCCT	SEQ ID NO:7	
	A4889G	M2F, CTGTCTCCCTCTGGTTACAGGAAGC	SEQ ID NO:8	204 bp
M2R, TTCCACCGTTGCAGCAGGATAGCC		SEQ ID NO:9		
C4887A	M2F, CTGTCTCCCTCTGGTTACAGGAAGC	SEQ ID NO:8	204 bp	
	M2R, TTCCACCGTTGCAGCAGGATAGCC	SEQ ID NO:9		
CYP2D6	G1934A	C, GCCTTCGCCAACCACTCCG	SEQ ID NO:10	334 bp
		D, AAATCCTGCTCTTCCGAGGC	SEQ ID NO:11	
A2637del	E, GATGAGCTGCTAACTGAGCCC	SEQ ID NO:12	268 bp	
		F, CCGAGAGCATACTCGGGAC		SEQ ID NO:13
CYP3A4	A-290G	3A4F2, TAGGTAAAGATCTGTAGGTGT	SEQ ID NO:14	266 bp
		3A4R1, GCTTCTCCACCTTGAAG	SEQ ID NO:15	

5 In particular, the present invention relates to
the design and utilization of oligonucleotide molecules
comprising a mutant allele of CYP2D6 which contains a
point mutation in at least one of the positions 1934,
or 2637. The point mutation at 1934 is adenine and the
10 whole oligonucleotide has the sequences shown in
Table 2. The point mutation at 2637 is a deletion of
an adenine and the whole oligonucleotide has the

sequences shown in Table 2. The invention relates to the design and utilization of oligonucleotide molecules comprising a wild-type (no mutation) allele of CYP2D6 which contains the normal nucleotide in at least one of the positions 1934 or 2637. The normal residue at 1934 is guanine and the whole oligonucleotide has the sequences shown in Table 2. The normal residue at 2637 is adenine and the whole oligonucleotide has the sequences shown in Table 2.

Table 2
Characteristics of allele-specific-oligonucleotide probes

Locus	Mutation	ASO	SEQ ID NO:	Hyb. T (°C)	Wash. (°C)
NAT2	T341C	Mut, ggtgaccaCtgacgg	SEQ ID NO:16	42	42
		Wt, ccgtcaAtggtcacc	SEQ ID NO:17	42	42
	C481T	Mut, attggtccaAgtac	SEQ ID NO:18	38	38
		Wt, gtacCtggaccaa	SEQ ID NO:19	38	38
	G590A	Mut, acctcAaacaattga	SEQ ID NO:20	37	37
		Wt, tcaattgttCgaggt	SEQ ID NO:21	37	37
	A803G	Wt, agtgctgaAaaatat	SEQ ID NO:22	37	40
		Mut, atatttCtcagcact	SEQ ID NO:23	42	37
	G857A	Mut, cctggtgatgAatcc	SEQ ID NO:24	42	42
		Wt, ggatCcatcaccagg	SEQ ID NO:25	42	55
CYP1A1	T6235C	Wt, tgagcccAggaggtg	SEQ ID NO:26	37	42
		Mut, cacctccCgggctca	SEQ ID NO:27	37	42
	A4889G	Mut, gggcaaCggctcac	SEQ ID NO:28	37	40
		Wt, gtgagaccAttgccc	SEQ ID NO:29	35	48
	C4887A	Wt, gtgagaCcattgccc	SEQ ID NO:30	35	48
		Mut, gtgagaAcattgccc	SEQ ID NO:31	37	38
CYP2D6	G1934A	Wt, ggcgtcCtgg	SEQ ID NO:32	37	40
		Mut, ggcgtcTgg	SEQ ID NO:33	37	40
	A2637del	Wt, gagcacaggatgacc	SEQ ID NO:34	37	46
		Mut, tgagcac-ggatgacc	SEQ ID NO:35	37	46
CYP3A4	A-290G	Wt, agagacaagggcaAgagag	SEQ ID NO:36	37	55
		Mut, agagacaagggcaGgagag	SEQ ID NO:37	37	55

Wt, wild-type sequence; mut, mutant sequence; uppercase characters indicate point mutation sites

5

In particular, the present invention relates to the design and utilization of oligonucleotide molecules comprising a mutant allele of CYP3A4 which contains a point mutation in the position -290. The point mutation at -290 is guanine and the whole oligonucleotide has the sequences shown in Table 2. The invention relates to the design and utilization of oligonucleotide molecules comprising a wild-type (no mutation)

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allele of CYP3A4 which contains the normal nucleotide in the position -290. The normal residue at -290 is adenine and the whole oligonucleotide has the sequences shown in Table 2.

5 In particular, the present invention relates to the design and utilization of oligonucleotide molecules comprising a mutant allele of NAT2 which contains a point mutation in at least one of the positions 341, 481, 590, 803 and 857. The point mutation at 341 is
10 cytosine and the whole oligonucleotide has the sequences shown in Table 2. The point mutation at 481 is thymine and the whole oligonucleotide has the sequences shown in Table 2. The point mutation at 590 is adenine and the whole oligonucleotide has the
15 sequences shown in Table 2. The point mutation at 803 is guanine and the whole oligonucleotide has the sequences shown in Table 2. The point mutation at 857 is adenine and the whole oligonucleotide has the sequences shown in Table 2. The invention relates to
20 the design and utilization of oligonucleotide molecules comprising a wild-type (no mutation) allele of NAT2 which contains the normal nucleotide in at least one of the positions 341, 481, 590, 803 and 857. The normal residue at 341 is thymine and the whole oligonucleotide
25 has the sequences shown in Table 2. The normal residue at 481 is cytosine and the whole oligonucleotide has the sequences shown in Table 2. The normal residue at 590 is guanine and the whole oligonucleotide has the sequences shown in Table 2. The normal residue at 803
30 is adenine and the whole oligonucleotide has the sequences shown in Table 2. The normal residue at 857 is guanine and the whole oligonucleotide has the sequences shown in Table 2.

Another aim of the present invention is to provide oligonucleotide molecules complementary to any of the oligonucleotide molecules described above.

Another aim of the present invention is to provide a diagnostic assay for determining CYP1A1 genotype of an individual which comprises isolating DNA from said individual; amplifying for CYP1A1 PCR fragment from said DNA, which includes at least one of the positions 4887, 4889, or 6235, thereby obtaining an amplified fragment; transferring said amplified fragments to membrane filter; hybridizing said membrane with allele-specific oligonucleotide corresponding to at least one of point mutation at the positions 4887, 4889, or 6235 and in parallel with the corresponding wild-type oligonucleotide; thereby determine the CYP1A1 genotype of said individual.

In a preferred embodiment of the invention, controls are run parallel to the above described reaction steps.

Another aim of the present invention is to provide a diagnostic assay for determining CYP1A1 genotype of an individual which comprises isolating DNA from said person; making a first and a second PCR primers wherein the first PCR primers is complementary to a region 5' to one of the point mutation sites at position 4887, 4889, or 6235; and the second PCR primer is complementary to a region 3' to the same one of the point mutation sites at position 4887, 4889, or 6235; amplifying the sequence in between the first and the second primers; thereby obtaining an amplified fragment. The whole sequence of the PCR primers is given in Table 1.

Another aim of the present invention is to provide a diagnostic assay for determining CYP2D6 genotype of an individual which comprises isolating DNA from

said person; making a first and a second PCR primers wherein the first PCR primers is complementary to a region 5' to one of the point mutation sites at position 1934, or 2637; and the second PCR primer is complementary to a region 3' to the same one of the point mutation sites at position 1934, or 2637; amplifying the sequence in between the first and the second primers; thereby obtaining an amplified fragment. The whole sequence of the PCR primers is given in Table 1.

10 Another aim of the present invention is to provide a diagnostic assay for determining CYP3A4 genotype of an individual which comprises isolating DNA from said person; making a first and a second PCR primers wherein the first PCR primers is complementary to a region 5' to the point mutation site at position -290; and the second PCR primer is complementary to a region 3' to the same one of the point mutation site at position -290; amplifying the sequence in between the first and the second primers; thereby obtaining an amplified fragment. The whole sequence of the PCR primers is given in Table 1.

20 Another aim of the present invention is to provide a diagnostic assay for determining NAT2 genotype of an individual which comprises isolating DNA from said person; making a first and a second PCR primers wherein the first PCR primers is complementary to a region 5' to one of the point mutation sites at position 341, 481, 590, 803 or 857; and the second PCR primer is complementary to a region 3' to the same one of the point mutation sites at position 341, 481, 590, 803 or 857; amplifying the sequence in between the first and the second primers; thereby obtaining an amplified fragment. The whole sequence of the PCR primers is given in Table 1.

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DNA isolation

Genomic DNA of the individual subject is isolated by the known methods in the art, such as phenol/chloroform extraction from tissue containing nucleated cells including white blood cells, mouth epithelial cells, liver, etc.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

Example 1

Genotyping of CYP1A1 polymorphisms

A DNA fragment of 899bp containing the point mutation T6235C was amplified in 20 ul containing 20ng of genomic DNA, 0.5 uM of primers M3F (5'GGCTGAGCAATCTGACCCTA; SEQ ID NO:6) and P80 (5'TAGGAGTCTTGTCTCATGCCT; SEQ ID NO:7), 200 uM dNTPs, 10mM Tris-HCl (pH 8.3), 2.5mM MgCl₂, 50 mM KCl, and 0.5U AmpliTaq DNA polymerase (Hoffman-LaRoche). PCR was carried out for 35 cycles of 30s at 94°C, 1 min at 63°C, and min at 72°C. Mutations A4889G and C4887A were detected by amplifying a 204bp fragment with primers M2F (5'CTGTCTCCCTCTGGTTACAGGAAGC; SEQ ID NO:8) and M2R (5'TTCCACCCGTTGCAGCAGGATAGCC; SEQ ID NO:9) as described above. These mutations were then used to define three distinct alleles (Fig. 1), CYP1A1*2A (presence of T6235C only), *2B (both T6235C and A4889G) and *4 (C4887A only).

Dot blots

Each PCR reaction was brought up to 100 µl with water, incubated at 94°C for 2 min and cooled on ice; the solution was then made 10xSSC (1xSSC corresponds to 1.5 M NaCl, 150 mM sodium citrate, pH 7) by addition of 100 µl 20xSSC. Hundred µl aliquots were then applied

in parallel onto a membrane filter to create two identical twin 48-dot blots. After rinsing with 100 μ l of 10xSSC the membranes were immersed in 1.5 M NaCl, 0.5 M NaOH for 10 min and in 1.5 M NaCl, 0.5 M Tris HCl pH 7.2 for 15 min DNA was fixed by drying the membranes and exposing them to 254 nm UV at the energy of 120,000 μ J/cm² in Stratalinker 1800 (Stratagene).

ASO-hybridization

Pentadecanucleotides were synthesized complementary to each allelic variant identified, to serve as ASO-probes for DNA typing by dot blot hybridization (Table 2).

Blots were pre-hybridized for 30 min (rotary oven) in 20 ml 1xSSPE (150 mM NaCl, 10 mM NaH₂PO₄, 1.1 mM EDTA, pH 7.4), 0.75 M NaCl, 70 mM Tris/HCl, pH 7.4 containing 1% SDS and 200 μ g/ml heparin, at hybridization temperature, T_H (see Table 2).

ASO probes may be labeled with any detectable label, including, without limitation, alkaline phosphatase, radioisotopes and biotin among others.

Preferably, ASO probes, 50 pmole, were 5'-labeled using γ -[³²P]-ATP (6,000 Ci/mmmole) and T4 kinase (Gibco BRL) to a specific activity of 1-3 x 10⁶ cpm/pmole (250,000-750,000 cpm/ng). Hybridization with the 0.8-2.0 pmole ASO probe (~2,000,000 cpm) was carried out for 40 min at T_H. Then the membranes were washed with 2xSSPE, 0.1% SDS for 10 min at room temperature, once or 2 times for 10 min at washing temperature (see Table 2) and exposed overnight at -80°C with a screen. Identical twin membranes were probed by the allelic ASOs and always read in parallel. This compensated, if necessary, for varying concentrations of the individual amplified DNA samples and, by the same token, for the variance in the probe activity. In addition, DNA samples of known allelic content served

as positive controls for the allelic probes. After stripping (5 min in boiling 0.5% SDS) the membranes were stored at room temperature (or at -20°C for longer periods of time) and reused (up to 12 times) for hybridization with other probes.

Example 2

Genotyping of CYP2D6 polymorphisms

The mutant CYP2D6*4 (G-to-A transition at position 1934) and CYP2D6*3 (a 1 bp deletion in position 2637) alleles (Fig. 2) were detected by PCR amplification using primers C/D (C, 5'GCCTTCGCCAACCCTCCG (SEQ ID NO:10); D, 5'AAATCCTGCTCTTCCGAGGC; SEQ ID NO:11), and primers E/F (E, 5'GATGAGCTGCTAACTGAGCCC; SEQ ID NO:12; F, 5'CCGAGAGCATACTCGGGAC; SEQ ID NO:13), respectively. PCR was carried out for 35 cycles of 30s at 94°C, 45s at 56°C (*3) or 60°C (*4), and 45s at 72°C in 20 ul containing 20ng of genomic DNA, 1.0 uM of each primer, 200 uM dNTPs, 10mM Tris-HCl (pH 8.3), 1.5mM MgCl₂, 50 mM KCl, and 0.5U AmpliTaq DNA polymerase (Hoffman-LaRoche).

Dot blots

Each PCR reaction was brought up to 100 µl with water, incubated at 94°C for 2 min and cooled on ice; the solution was then made 10xSSC (1xSSC corresponds to 1.5 M NaCl, 150 mM sodium citrate, pH 7) by addition of 100 µl 20xSSC. Hundred µl aliquots were then applied in parallel onto a membrane filter to create two identical twin 48-dot blots. After rinsing with 100 µl of 10xSSC the membranes were immersed in 1.5 M NaCl, 0.5 M NaOH for 10 min and in 1.5 M NaCl, 0.5 M Tris HCl pH 7.2 for 15 min DNA was fixed by drying the membranes and exposing them to 254 nm UV at the energy of 120,000 µJ/cm² in Stratalinker 1800 (Stratagene).

ASO-hybridization

Pentadecanucleotides were synthesized complementary to each allelic variant identified, to serve as ASO-probes for DNA typing by dot blot hybridization
5 (Table 2).

Blots were pre-hybridized for 30 min (rotary oven) in 20 ml 1xSSPE (150 mM NaCl, 10 mM NaH₂PO₄, 1.1 mM EDTA, pH 7.4), 0.75 M NaCl, 70 mM Tris/HCl, pH 7.4 containing 1% SDS and 200 µg/ml heparin, at hybridiza-
10 tion temperature, T_H (see Table 2).

ASO probes may be labeled with any detectable label, including, without limitation, alkaline phosphatase, radioisotopes and biotin among others.

Preferably, ASO probes, 50 pmole, were 5'-
15 labeled using γ-[³²P]-ATP (6,000 Ci/mmole) and T4 kinase (Gibco BRL) to a specific activity of 1-3 x 10⁶ cpm/pmole (250,000-750,000 cpm/ng). Hybridization with the 0.8-2.0 pmole ASO probe (~2,000,000 cpm) was carried out for 40 min at T_H. Then the membranes were
20 washed with 2xSSPE, 0.1% SDS for 10 min at room temperature, once or 2 times for 10 min at washing temperature (see Table 2) and exposed overnight at -80°C with a screen. Identical twin membranes were probed by the allelic ASOs and always read in parallel. This
25 compensated, if necessary, for varying concentrations of the individual amplified DNA samples and, by the same token, for the variance in the probe activity. In addition, DNA samples of known allelic content served as positive controls for the allelic probes. After
30 stripping (5 min in boiling 0.5% SDS) the membranes were stored at room temperature (or at -20°C for longer periods of time) and reused (up to 12 times) for hybridization with other probes.

Example 3

Genotyping of CYP3A4 polymorphism

The mutant allele (Fig. 3) CYP3A4*2 (A-290G) was
5 detected by PCR amplification using the PCR primers
3A4F2 (5'TAGGTAAAGATCTGTAGGTGT3'; SEQ ID NO:14) and
3A4R1 (5'GCTTCTCCACCTTGGAAG3'; SEQ ID NO:15). PCR was
carried out for 35 cycles of 30s at 94C, 30s at 56°C and
30s at 72°C in 25 ul containing 15 ng of genomic DNA,
10 0.25uM of each primers, 100u m dNTPs, 10mM Tris-HCl (pH
8.3), 1,5 mM MgCl₂, 50 mM KCl and 0.2U ampliTaQ DNA
polymerase (Hoffman-Laroche).

Dot blots

Each PCR reaction was brought up to 100 µl with
15 water, incubated at 94°C for 2 min and cooled on ice;
the solution was then made 10xSSC (1xSSC corresponds to
1.5 M NaCl, 150 mM sodium citrate, pH 7) by addition of
100 µl 20xSSC. Hundred µl aliquots were then applied
in parallel onto a membrane filter to create two iden-
20 tical twin 48-dot blots. After rinsing with 100 µl of
10xSSC the membranes were immersed in 1.5 M NaCl, 0.5 M
NaOH for 10 min and in 1.5 M NaCl, 0.5 M Tris HCl pH
7.2 for 15 min DNA was fixed by drying the membranes
and exposing them to 254 nm UV at the energy of
25 120,000 µJ/cm² in Stratalinker 1800 (Stratagene).

ASO-hybridization

Pentadecanucleotides were synthesized comple-
mentary to each allelic variant identified, to serve as
ASO-probes for DNA typing by dot blot hybridization
30 (Table 2).

Blots were pre-hybridized for 30 min (rotary
oven) in 20 ml 1xSSPE (150 mM NaCl, 10 mM NaH₂PO₄, 1.1 mM
EDTA, pH 7.4), 0.75 M NaCl, 70 mM Tris/HCl, pH 7.4
containing 1% SDS and 200 µg/ml heparin, at hybridiza-
35 tion temperature, T_H (see Table 2).

ASO probes may be labeled with any detectable label, including, without limitation, alkaline phosphatase, radioisotopes and biotin among others.

Preferably, ASO probes, 50 pmole, were 5'-
5 labeled using γ -[³²P]-ATP (6,000 Ci/mmole) and T4 kinase (Gibco BRL) to a specific activity of 1-3 x 10⁶ cpm/pmole (250,000-750,000 cpm/ng). Hybridization with the 0.8-2.0 pmole ASO probe (~2,000,000 cpm) was carried out for 40 min at T_H. Then the membranes were
10 washed with 2xSSPE, 0.1% SDS for 10 min at room temperature, once or 2 times for 10 min at washing temperature (see Table 2) and exposed overnight at -80°C with a screen. Identical twin membranes were probed by the allelic ASOs and always read in parallel. This
15 compensated, if necessary, for varying concentrations of the individual amplified DNA samples and, by the same token, for the variance in the probe activity. In addition, DNA samples of known allelic content served as positive controls for the allelic probes. After
20 stripping (5 min in boiling 0.5% SDS) the membranes were stored at room temperature (or at -20°C for longer periods of time) and reused (up to 12 times) for hybridization with other probes.

25

Example 4

Genotyping of NAT2 polymorphisms

A 1211 bp fragment containing the whole NAT2 coding region was amplified with primers P100 (5'GTCACACGAGGAAATCAAATGC3'; SEQ ID NO:1) and P56
30 (5'GTTTTCTAGCATGAATCACTCTGC3'; SEQ ID NO:3). PCR was carried out for 35 cycles of 30s at 94°C, 1 min at 62°C and 2 min at 72°C in 20 ul containing 20 ng of genomic DNA, 0.5uM of each primer, 200 uM of each dNTPs, 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5 mM MgCl₂ and 1.0 U
35 ampliTag DNA polymerase (Hoffman-Laroche). In problem-

atic cases, the same PCR product was used for two nested PCR. One was carried out with P100 (SEQ ID NO:1) and R1 (5'ACCCAGCATCGACAATGTAATTCCTGCCCTCA3'; SEQ ID NO:2), for 35 cycles of 30s at 94°C, 30s at 62°C and 45s at 72°C in 20 ul containing 1 ul of first-step PCR product, 0.5uM of each primers, 200 uM of each dNTPs, 1.5mM MgCl₂, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 0.5U ampliTaQ DNA polymerase (Hoffman-Laroche). In the second nested PCR was performed with 0.5uM of primers P87 (5'CCTGGACCAAATCAGGAGAG3'; SEQ ID NO:4) and P90 (5'ACACAAGGGTTTATTTTGTTC3'; SEQ ID NO:5). These mutations are then used to define six distinct alleles (Fig. 4), NAT2*4 (no mutation), NAT2*5A (presence of mutations at positions 341 and 481), NAT2*5B (presence of mutations at positions 341, 481 and 803), NAT2*5C (presence of mutations at positions 341 and 803), NAT*6A (presence of mutations at positions 282 and 590) and NAT*7B (presence of mutations at positions 282 and 857).

20 Dot blots

Each PCR reaction was brought up to 100 µl with water, incubated at 94°C for 2 min and cooled on ice; the solution was then made 10xSSC (1xSSC corresponds to 1.5 M NaCl, 150 mM sodium citrate, pH 7) by addition of 100 µl 20xSSC. Hundred µl aliquots were then applied in parallel onto a membrane filter to create two identical twin 48-dot blots. After rinsing with 100 µl of 10xSSC the membranes were immersed in 1.5 M NaCl, 0.5 M NaOH for 10 min and in 1.5 M NaCl, 0.5 M Tris HCl pH 7.2 for 15 min DNA was fixed by drying the membranes and exposing them to 254 nm UV at the energy of 120,000 µJ/cm² in Stratalinker 1800 (Stratagene).

30 ASO-hybridization

Pentadecanucleotides were synthesized complementary to each allelic variant identified, to serve as

ASO-probes for DNA typing by dot blot hybridization (Table 2).

Blots were pre-hybridized for 30 min (rotary oven) in 20 ml 1xSSPE (150 mM NaCl, 10 mM NaH₂PO₄, 1.1 mM EDTA, pH 7.4), 0.75 M NaCl, 70 mM Tris/HCl, pH 7.4
5 containing 1% SDS and 200 µg/ml heparin, at hybridization temperature, T_H (see Table 2).

ASO probes may be labeled with any detectable label, including, without limitation, alkaline
10 phosphatase, radioisotopes and biotin among others.

Preferably, ASO probes, 50 pmole, were 5'-labeled using γ-[³²P]-ATP (6,000 Ci/mmol) and T4 kinase (Gibco BRL) to a specific activity of 1-3 x 10⁶ cpm/pmole (250,000-750,000 cpm/ng). Hybridization with
15 the 0.8-2.0 pmole ASO probe (~2,000,000 cpm) was carried out for 40 min at T_H. Then the membranes were washed with 2xSSPE, 0.1% SDS for 10 min at room temperature, once or 2 times for 10 min at washing temperature (see Table 2) and exposed overnight at -80°C
20 with a screen. Identical twin membranes were probed by the allelic ASOs and always read in parallel. This compensated, if necessary, for varying concentrations of the individual amplified DNA samples and, by the same token, for the variance in the probe activity. In
25 addition, DNA samples of known allelic content served as positive controls for the allelic probes. After stripping (5 min in boiling 0.5% SDS) the membranes were stored at room temperature (or at -20°C for longer periods of time) and reused (up to 12 times) for
30 hybridization with other probes.

RESULTS AND DISCUSSION

To facilitate the analysis of mutations in the carcinogen metabolizing genes CYP1A1, CYP2D6, CYP3A4 and NAT2 (Figs. 1-4) we have developed a PCR-ASO system
35 for efficient screening of at least 11 mutations.

The numbering of nucleotide positions in Fig. 1 is as in Cascorbi et al. (1996b). Wild type, WT, and mutant variants are shown below where the effect of mutation on the integrity of the message or the protein coding (if any) is indicated. CYP1A1 represents WT; substitution T/C (position 6235) in 3' flanking region of the CYP1A1 gene defines CYP1A1*2A allele and in phase with the A/G base substitution at position 4889, causing substitution of Ile to Val (codon 462, it defines CYP1A1*2B allele; C/A substitution at position 4887, leading to replacement of Thr by Asp (codon 461), defines CYP1A1*4 allele.

The numbering of nucleotide positions in Fig. 2 is as in Sachse et al. (1997). Wild type, WT, and mutant variants are shown below where the effect of mutation on the integrity of the message or the protein coding (if any) is indicated. CYP2D6*1 is a wild type; CYP2D6*4 allele is defined by G/A mutation at site 1934 (last position of intron 3) which leads to an abnormal splicing, while a deletion at the position 2637 (CYP2D6*3) causes a frameshift at the Arg codon 259.

The numbering of nucleotide positions in Fig. 3 is as in Hashimoto et al. (Hashimoto H et al., 1993, *Eur J Biochem.* **218**:585-595). CYP3A4*1 is a wild type; CYP3A4*2 allele is defined by A/G mutation at site -290 in the 5'untranslated region.

The numbering of nucleotide positions in Fig. 4 is as in Cascorbi et al. (1996a). WT and mutant variants are shown below where the effect of mutation on the protein coding (if any) is indicated. NAT*5A allele is characterized by T/C substitution at position 341 causing Ile/Thr amino-acid replacement at codon 114 which is accompanied by silent C/T mutation at the position 481. An A/G base substitution at position 803 (Lys/Arg amino-acid substitution at codon 268) on

NAT*5A background defines NAT2*5B allele. NAT2*5C differs from NAT2*5B by the absence of mutation at the 481 site. NAT2*6A allele is characterized by G/A substitution at the position 590 causing Arg/Gln replacement at codon 197. NAT2*7B allele is defined by G/A substitution at position 857 causing Gly/Glu replacement at codon 286. DNA fragments including sites of polymorphisms within CYP1A1, CYP2D6, CYP3A4 and NAT2 loci were amplified by PCR, dot-blotted and subsequently hybridized with ASOs. Representative hybridization blots are illustrated in Fig. 4.

Positive hybridization signals with mutant probes indicate the presence of mutations, absence of the hybridization signal with the wild-type probe indicates mutant homozygous. A heterozygous individual will be illustrated by positive hybridization signals both with mutant and wild-type probes, whereas the absence of signal with the mutant ASO but a positive signal with the wild-type probe indicates a wild-type homozygous status (Fig. 5). Two 48-dots sister blots were probed with oligonucleotide-probes, specific for the wild type (left panels) or the mutant (right panels) alleles (Fig. 5). The name of the locus as well as the WT and MUT allele-specific oligonucleotide probes are indicated (only representative columns and rows from the twin blots are shown).

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be

applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. An isolated oligonucleotide molecule comprising a sequence hybridizing to a gene encoding xenobiotics metabolizing enzymes CYP1A1, CYP2D6, CYP3A4, or NAT2, wild type and mutant alleles thereof; wherein said sequence is sufficiently complementary to said gene to hybridize therewith.
2. An isolated oligonucleotide molecule comprising a mutant allele of CYP1A1, which contains a point mutation at one position selected from the group consisting of position 4887, 4889, 5639 and 6235.
3. An isolated oligonucleotide molecule of claim 2, wherein said mutation is selected from the group consisting of an adenine at position 4887, a guanine at position 4889, a cytosine at position 5639 and a cytosine at position 6235.
4. An isolated oligonucleotide molecule of claim 3, wherein said mutant oligonucleotide molecule has a nucleic acid sequence as set forth in SEQ ID NOS:27, 28 and 31.
5. An isolated oligonucleotide molecule comprising a wild-type allele of CYP1A1, which contains a normal nucleotide at one position selected from the group consisting of position 4887, 4889, 5639 and 6235.
6. An isolated oligonucleotide molecule as claimed in 5, wherein said normal nucleotide is selected from the group consisting of a cytosine at position 4887, an adenine at position 4889, a thymine at position 5639 and a thymine at position 6235.

7. An isolated oligonucleotide molecule as claimed in 6, wherein said wild type oligonucleotide molecule has a nucleic acid sequence as set forth in SEQ ID NOS:26, 29 and 30.

8. An isolated oligonucleotide molecule comprising a mutant allele of CYP3A4, contains a point mutation at position 290.

9. An isolated oligonucleotide molecule of claim 8, wherein said mutation is a guanine.

10. An isolated oligonucleotide molecule of claim 9, wherein said mutant oligonucleotide molecule has a nucleic acid sequence as set forth in SEQ ID NO:37.

11. An isolated oligonucleotide molecule comprising a wild-type allele of CYP3A4, which contains a normal nucleotide at position -290.

12. An isolated oligonucleotide molecule as claimed in 11, wherein said normal nucleotide is an adenine.

13. An isolated oligonucleotide molecule as claimed in 12, wherein said wild type oligonucleotide molecule has a nucleic acid sequence as set forth in SEQ ID NO:36.

14. An isolated oligonucleotide molecule comprising a mutant allele of CYP2D6, which contains a point mutation at one position selected from the group consisting of position 1934, 2637, 188, 212, 226, 971, 1111, 1726, 1795, 1846, 2064, 2701-2703, 2702-2704, and 3023.

15. An isolated oligonucleotide molecule of claim 14, wherein said mutation is selected from the group consisting of an adenine at position 1934, a deletion at position 2637, a thymine at position 188, an adenine at position 212, an insertion of a thymine at position 226, a cytosine at position 971, a thymine at position 1111, a cytosine at position 1726, a deletion of a thymine at position 1795, a thymine at position 1846, an adenine at position 1846, an adenine at position 2064, a three-nucleotide deletion (adenine-guanine-adenine) at positions 2701-to-2703, a three-nucleotide deletion (guanine-adenine-guanine) at positions 2702-to-2704, and a cytosine at position 3023.

16. An isolated oligonucleotide molecule of claim 15, wherein said mutant oligonucleotide molecule has a nucleic acid sequence as set forth in SEQ ID NOS:33 and 35.

17. An isolated oligonucleotide molecule comprising a wild-type allele of CYP2D6, which contains a normal nucleotide at one position selected from the group consisting of position 1934, 2637, 188, 212, 226, 971, 1111, 1726, 1795, 1846, 2064, 2701-2703, 2702-2704, and 3023.

18. An isolated oligonucleotide molecule as claimed in 17, wherein said normal nucleotide is selected from the group consisting of a guanine at position 1934, an adenine at position 2637, a cytosine at position 188, a cytosine at position 212, no insertion at position 226, a guanine at position 971, a cytosine at position 1111, a guanine at position 1726, a thymine at position 1795,

a guanine at position 1846, a guanine at position 2064, an adenine-guanine-adenine at positions 2701-to-2703, a guanine-adenine-guanine at positions 2702-2704, and an adenine at position 3023.

19. An isolated oligonucleotide molecule as claimed in 18, wherein said wild type oligonucleotide molecule has a nucleic acid sequence as set forth in SEQ ID NOS:32 and 34.

20. An isolated oligonucleotide molecule comprising a mutant allele of NAT2, which contains a point mutation at one position selected from the group consisting of position 341, 481, 590, 803, 857, 191, 282, 434, and 845.

21. An isolated oligonucleotide molecule of claim 20, wherein said mutation is selected from the group consisting of a cytosine at position 341, a thymine at position 481, an adenine at position 590, a guanine at position 803, an adenine at position 857, an adenine at position 191, a thymine at position 282, a cytosine at position 434, and a cytosine at position 845.

22. An isolated oligonucleotide molecule of claim 21, wherein said mutant oligonucleotide molecule has a nucleic acid sequence as set forth in SEQ ID NOS:16, 18, 20, 23, and 24.

23. An isolated oligonucleotide molecule comprising a wild-type allele of NAT2, which contains a normal nucleotide at one position selected from the group consisting of position 341, 481, 590, 803, 857, 191, 282, 434, and 845.

24. An isolated oligonucleotide molecule as claimed in 23, wherein said normal nucleotide is selected from the group consisting of a thymine at position 341, a cytosine at position 481, a guanine at position 590, an adenine at position 803, a guanine at position 857, a guanine at position 191, a cytosine at position 282, an adenine at position 434, and an adenine at position 845.

25. An isolated oligonucleotide molecule as claimed in 24, wherein said wild type oligonucleotide molecule has a nucleic acid sequence as set forth in SEQ ID NOS:17, 19, 21, 22, and 25.

26. An oligonucleotide molecule complementary to any of the oligonucleotide molecules identified as SEQ ID NOS:16-37.

27. A diagnostic assay for determining genetic variants in CYP1A1, CYP3A4, CYP2D6 or NAT2 gene in a subject, which comprises the steps of:

- a) obtaining a genomic DNA sample of said subject;
- b) using the DNA sample of step a), amplifying a fragment comprising a polymorphic site of at least one of CYP1A1, CYP3A4, CYP2D6 and NAT2 genes;
- c) hybridizing the amplified fragment of step b) with allele-specific oligonucleotides (ASO) probes corresponding to wild type and variant alleles to determine CYP1A1, CYP3A4, CYP2D6 or NAT2 genotype of said subject.

28. The diagnostic assay of claim 27, wherein amplifying step b) is with PCR primers set forth in Table 1.

29. The diagnostic assay of claim 27, which further comprises a step i) before step c) consisting in subjecting the amplified fragment of step b) to Southern dot blot transfer on membrane, and wherein step c) is effected by hybridizing the dot blots with the oligonucleotide probes.

30. The diagnostic assay of claim 28, wherein a labeled ASO probe is used in step c) and is selected from the sequences set forth in Table 2 and hybridizes under stringent conditions (Table 2).

31. The diagnostic assay of claim 30, wherein for CYP1A1 fragment from said DNA of step b) includes at least one of position 4887, 4889, 5639 or 6235; and the ASO probes of step d) correspond to at least one of the point mutations at positions 4887, 4889, 5639 or 6235 and to wild-type sequences thereof.

32. The diagnostic assay of claim 30, wherein for CYP2D6 fragment from said DNA of step b) includes at least one of position 1934, 2637, 188, 212, 226, 971, 1111, 1726, 1795, 1846, 2064, 2701-2703, 2702-2704, or 3023; and the ASO probes of step d) correspond to at least one of the point mutations at positions 1934, 2637, 188, 212, 226, 971, 1111, 1726, 1795, 1846, 2064, 2701-2703, 2702-2704, or 3023 and to wild-type sequences thereof.

33. The diagnostic assay of claim 30, wherein for CYP3A4 fragment from said DNA of step b) includes at

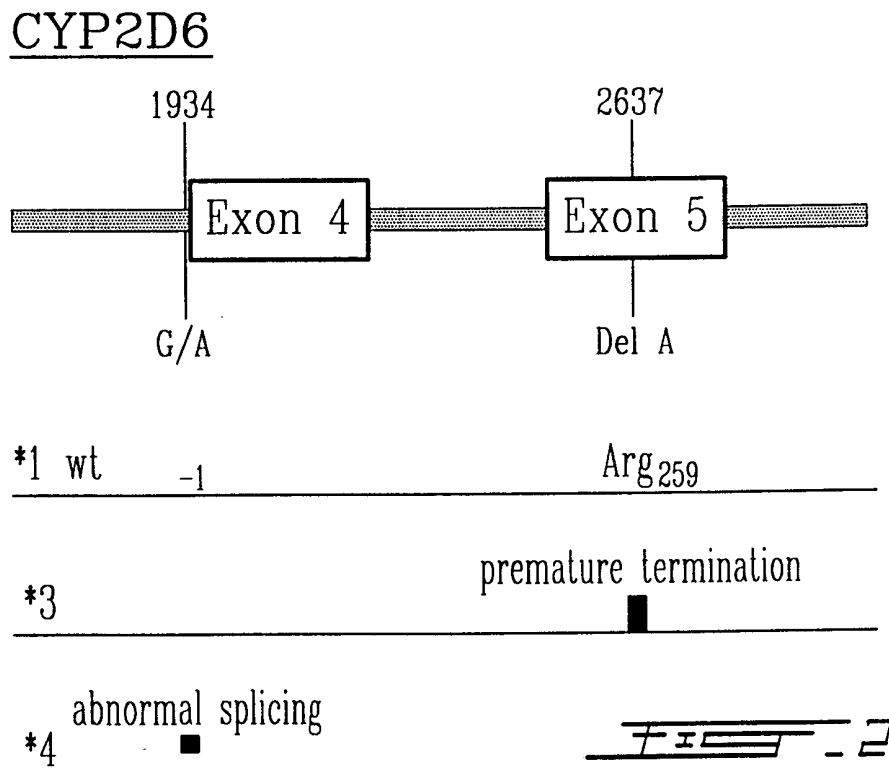
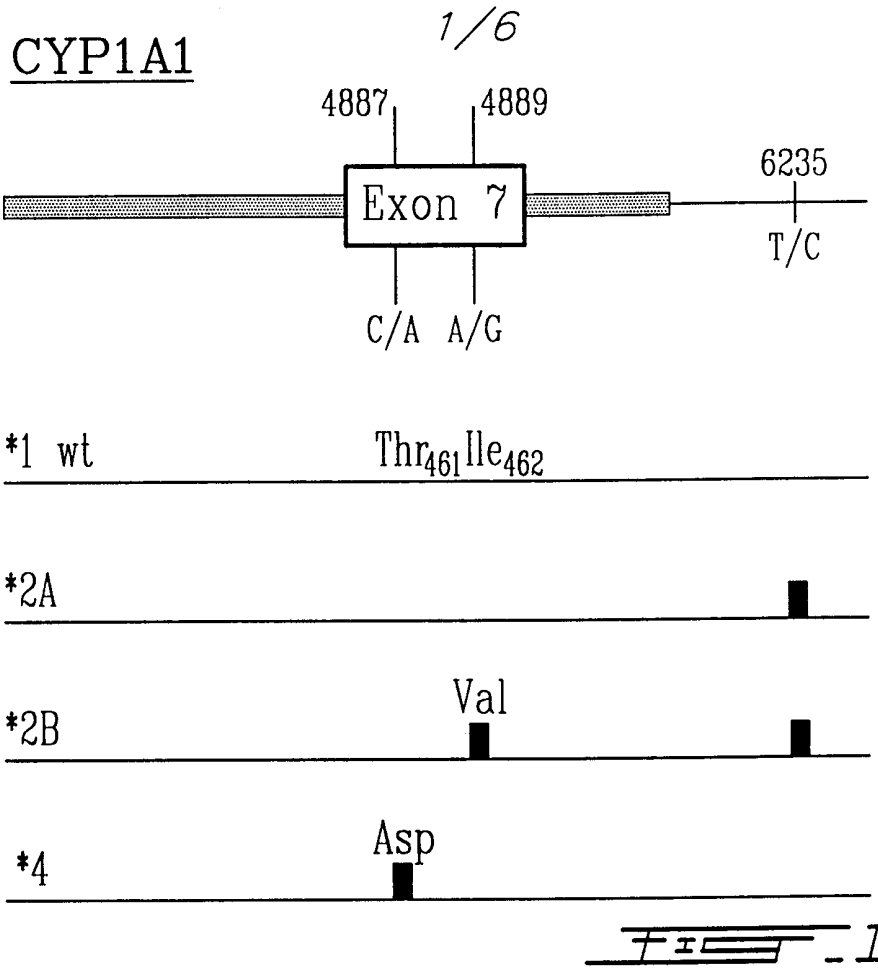
least position 290; and the ASO probes of step d) correspond to at least a point mutation at position 290 and to wild-type sequences thereof.

34. The diagnostic assay of claim 30, wherein for NAT2 fragment from said DNA of step b) includes at least one of position 341, 481, 590, 803, 857, 191, 282, 434, or 845; and the ASO probes of step d) correspond to at least one of the point mutations at positions 341, 481, 590, 803, 857, 191, 282, 434, or 845 and to wild-type sequences thereof.

35. A diagnostic kit for determining DNA variants in CYP1A1, CYP3A4, CYP2D6 or NAT2 gene in a subject, which comprises:

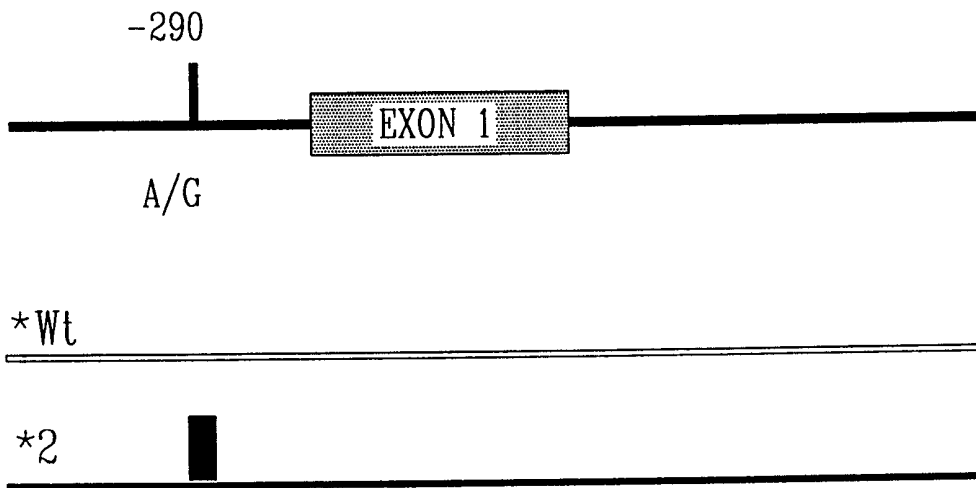
a) at least one of the PCR primers set forth in SEQ ID NOS:1-15; and

b) at least one of the ASO probes set forth in SEQ ID NOS:16-37.



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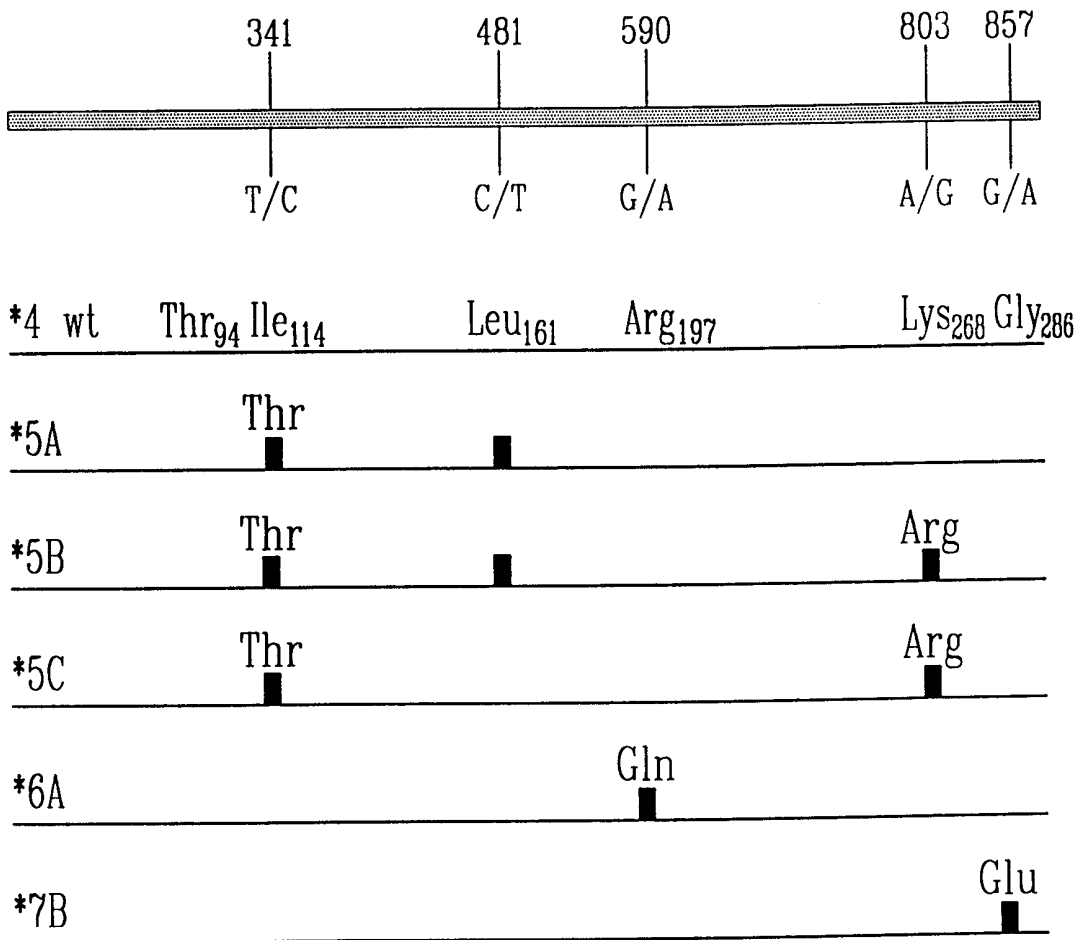
CYP3A4



~~FIG. 3~~

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NAT 2



~~FIG. 4~~

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CYP1A1

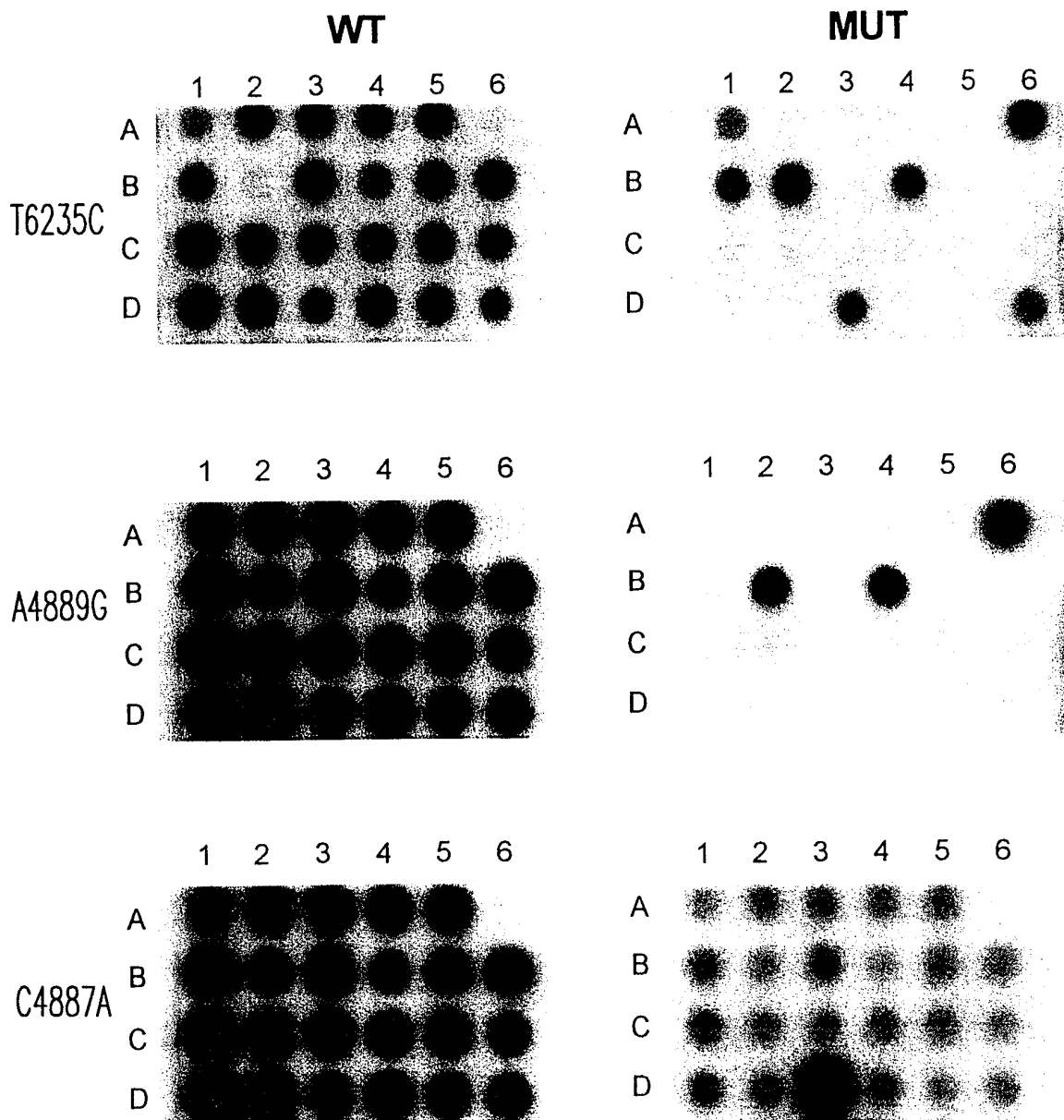
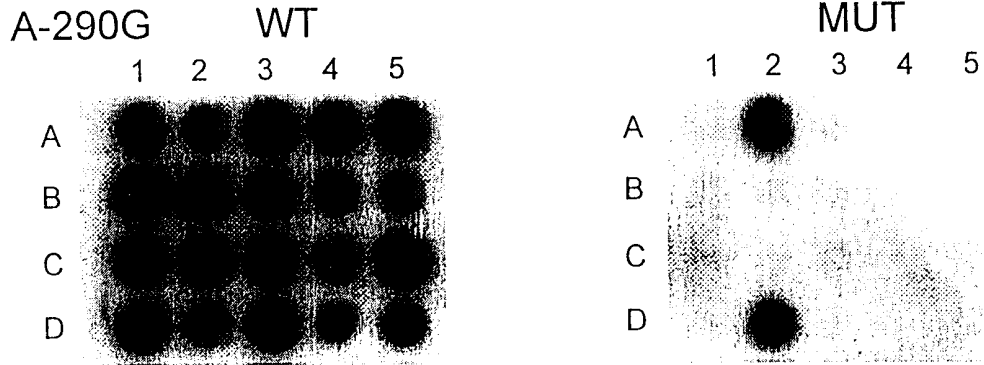


FIG. 5A

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CYP3A4



CYP2D6

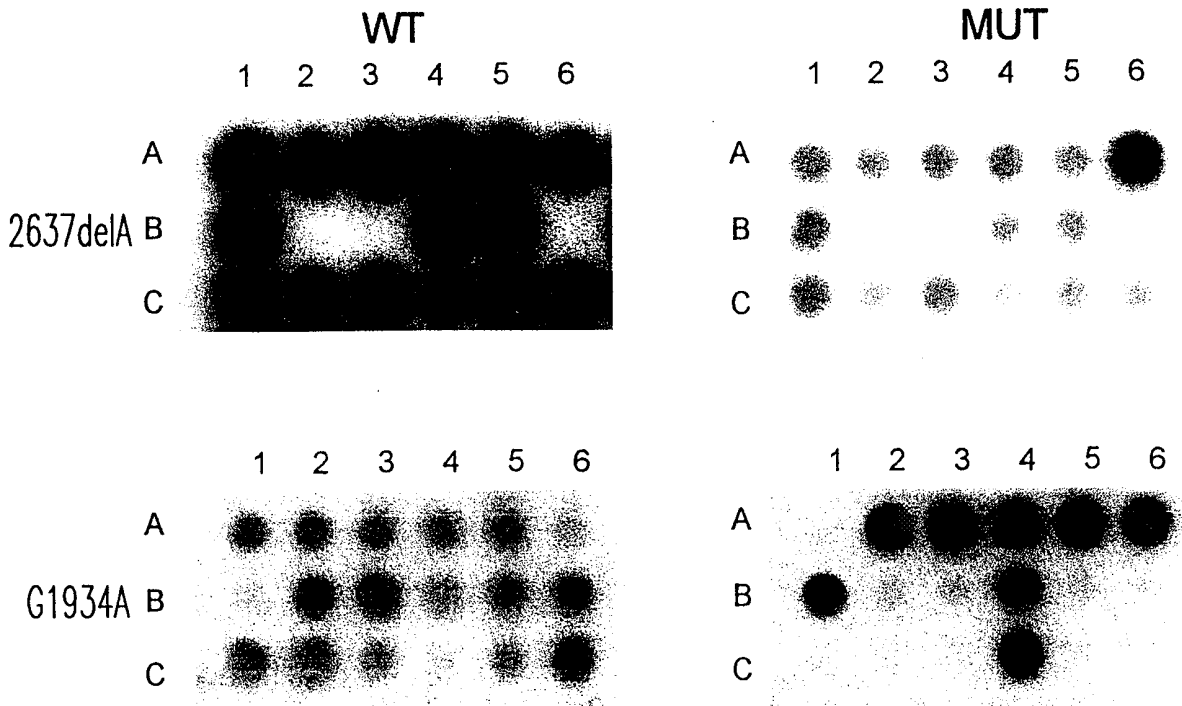
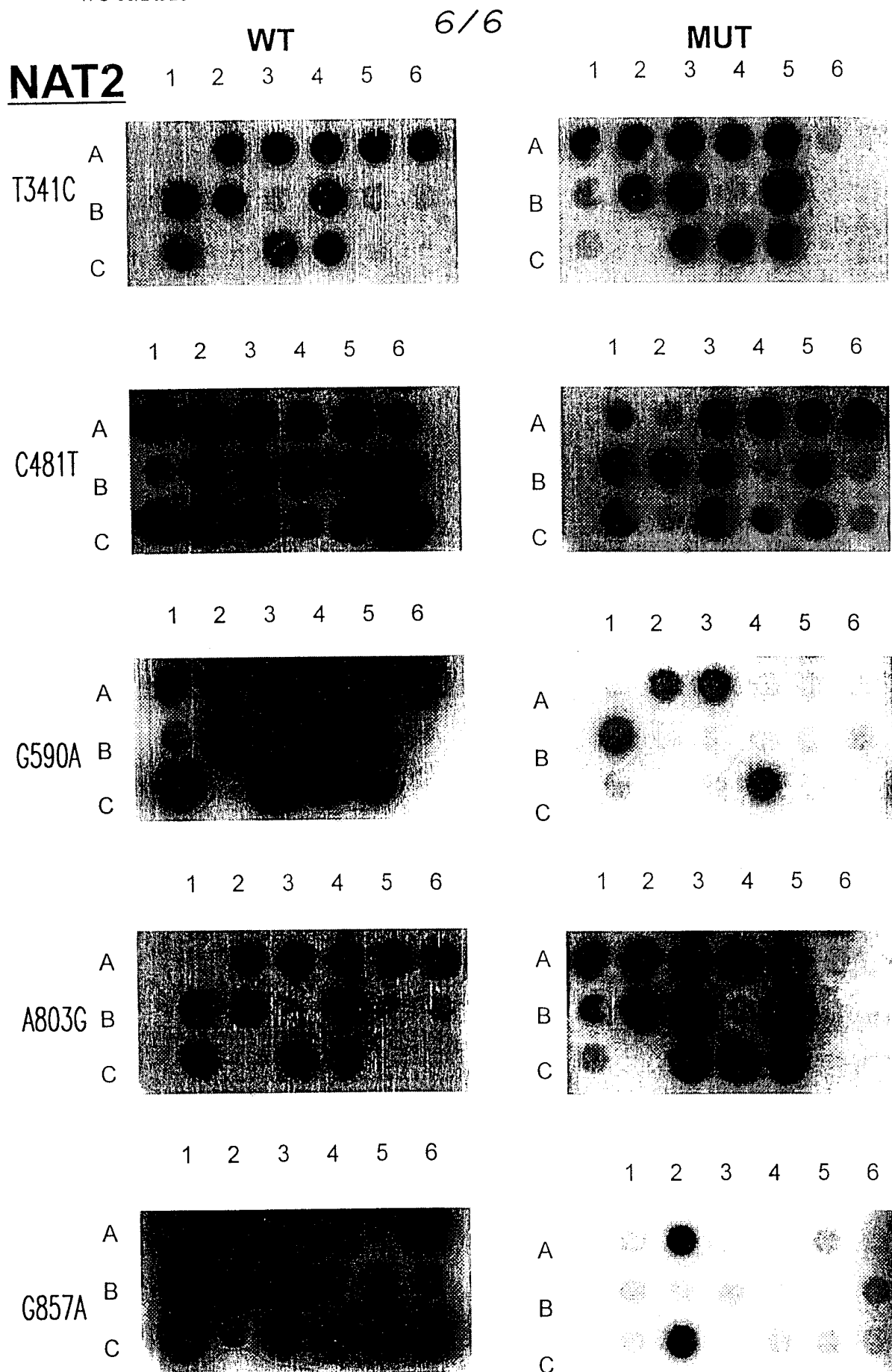


FIG. 5B



FIS-5C

SEQUENCE LISTING

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SINNETT, Daniel
LABUDA, Damian

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INTERNATIONAL SEARCH REPORT

Intern. Patent Application No

PCT/CA 99/00982

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 463 395 A (HOFFMANN LA ROCHE) 2 January 1992 (1992-01-02) the whole document	1, 14-21, 23, 24, 27, 35
X	WO 91 10745 A (IMP CANCER RES TECH) 25 July 1991 (1991-07-25) see whole doc. esp. claims and figures	1, 14, 27, 35
X	EP 0 759 476 A (OTSUKA PHARMA CO LTD) 26 February 1997 (1997-02-26) see claims and figures	1, 14, 17, 27, 35
P, X	WO 99 13106 A (AXYS PHARM INC) 18 March 1999 (1999-03-18) the whole document	1, 8-13, 26, 27, 33, 35

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Patent family members are listed in annex.

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Date of the actual completion of the international search

25 February 2000

Date of mailing of the international search report

08/03/2000

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	<p>KRAJINOVIC M. ET AL.,: "Susceptibility to childhood acute lymphoblastic leukemia: influence of CYP1A1, CYP2d6, GSTM1 and GSTM1 genetic polymorphisms" BLOOD, vol. 93, no. 5, - 1 March 1999 (1999-03-01) pages 1496-1501, XP002131547 the whole document</p> <p>_____</p>	<p>1-3,5,6, 14,15, 17,18, 27,35</p>
A	<p>genotyping of polymorphisms in xenobiotic metabolism as a predictor of disease susceptibility DAly A. et al., Enviromental health perspectives, 1994,102 suppl.9, 55-61 XP002131554 the whole document</p> <p>_____</p>	

INTERNATIONAL SEARCH REPORT

information on patent family members

Intern. Patent Application No

PCT/CA 99/00982

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0463395 A	02-01-1992	AU 645091 B	06-01-1994
		CA 2045012 A	23-12-1991
		JP 5211895 A	24-08-1993
		NZ 238610 A	25-11-1993
		US 5648482 A	15-07-1997
		US 5844108 A	01-12-1998
		DE 69126064 D	19-06-1997
		DE 69126064 T	28-08-1997
		ES 2103756 T	01-10-1997
WO 9110745 A	25-07-1991	AT 140488 T	15-08-1996
		AU 642705 B	28-10-1993
		AU 7179191 A	05-08-1991
		CA 2071636 A	19-07-1991
		DE 69120936 D	22-08-1996
		DE 69120936 T	20-02-1997
		EP 0511262 A	04-11-1992
		GB 2256271 A,B	02-12-1992
		JP 5503845 T	24-06-1993
		US 5981174 A	09-11-1999
		EP 0759476 A	26-02-1997
CA 2189638 A	16-11-1995		
CN 1151763 A	11-06-1997		
WO 9530772 A	16-11-1995		
WO 9913106 A	18-03-1999	AU 9128798 A	29-03-1999