D. 考察

今回の検討で、唾液検体を適切な方法で処理を行うと、PCR反応系へ唾液処理駅を直接添加可能であることが明らかになった。実際にこの方法を用い、唾液を検体としてCASSOH法を行うと遺伝子診断が可能であった。特にDNA精製を行うことなくPCR反応やCASSOH検出を行えるため、CASSOH法による遺伝子検査の迅速化に寄与する。

更に、唾液は、採取に痛みを伴わず、医療関係者でなくても可能である、感染のリスクが小さい、などの利点を有する。この事は、特に血液採取に技術と時間を要する小児に対し、オーダーメイド医療を行っていくうえで大きな進歩になると考えられる。

E. 結論

唾液を約10分間程度の処理で、PCR反応系へ直接添加することを可能にするプロトコールを確立した。唾液検体をこのプロトコールで処理することにより、CASSOH法による遺伝子検査の迅速化を達成した。

F. 健康危険情報

特になし

G. 研究発表

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H. 知的財産権の出願・登録状況

なし

厚生労働科学研究費補助金(創薬基盤推進研究事業)

分担研究報告書

CASSOH法の薬物代謝酵素遺伝子多型検査への応用

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研究要旨

CASSOH法を利用した遺伝子診断法の感度と特異性を向上させるために、ビオチン標識プローブと競合プローブの鎖長を変化させ、第一化学薬品製SNP検出ストリップに最も適した組合せを決定した。チオプリンメチルトランスフェラーゼ(TPMT)遺伝子のSNP(TPMT*3C(719A>G))検出系では、従来、野生型検出ビオチンプローブ及び競合プローブは18mer、変異型検出ビオチンプローブ及び競合プローブは17merの合成オリゴヌクレオチドを用いていた。今回、12～20merのプローブをそれぞれ合成し、最も感度良く、特異的に検出できる組合せが従来から用いてきた野生型で18mer、変異型で17merのプローブであることが判明した。また、CYP2C19遺伝子の2種のSNP検出系(CYP2C19*2(681G>A)及びCYP2C19*3(636G>A))でも、同様にプローブ鎖長の最適化ができた。また、CASSOH法によるTPMT*3C検出系においては、唾液由来のDNAを用いた場合でも迅速な検出が可能であった。

A. 研究目的

近年、薬剤反応性遺伝子における一塩基多型(SNP)解析に基づき、レスポンダー・ノンレスポンダーの識別や副作用予測を行うことで、患者個々に適した投与薬剤の用法・用量の選択が可能になると考えられている。現在国内外において、種々の薬剤反応性遺伝子のSNPと投与薬物の血中濃度・治療効果、副作用発現等の相関性に関する情報が積み上げられている。しかし、薬物療法に影響を与えるSNPを有する患者に対して、臨床レベルでその診断を行い、薬物の適正使用を行っている施設は非常に少ないと言える。今回、これまでに我々が構築してきた薬剤反応性に関与する遺伝子多型診断法(CASSOH法)のブラッシュアップを継続して行った。対象とする遺伝子は、TPMT(TPMT*3C(719A>G))及びCYP2C19(CYP2C19*2(681G>A)及びCYP2C19*3(636G>A))とした。検出法のブラッシュアップでは、検出プローブの鎖長を変化させ、最も感度良く、正確にSNPを検出できる組合せを特定した。また、DNA源として末梢血以外に唾液を検討した。

B. 研究方法

TPMT (TPMT*3C(719A>G))及びCYP2C19

(CYP2C19*2(681G>A) 及び CYP2C19*3(636G>A))をCASSOH法により検出し、最適なプローブ条件の決定を行った。方法は、まずSNPを挟む領域で通常のPCRを行った。ただし、その際に一方のプライマーの5'端をDIG(ジゴキシゲニン)でラベルした。また、反応液中にはDIGプライマーとは逆鎖に作成した5'及び3'端ビオチンラベル対立遺伝子特異的(野生型及び変異型検出)オリゴヌクレオチドプローブ(SNP部位は中央付近に来るように)を加えた。さらに非特異的なプローブのハイブリダイゼーションを阻害するため、野生型検出ビオチンプローブに対しては3'端リン酸ラベル変異型競合プローブを、変異型検出ビオチンプローブには3'端リン酸ラベル野生型競合プローブを添加した。なお、これらのプローブは9～20merのものを設計した。次にPCR後、高温でPCR産物を一本鎖にし、続いて温度を段階的に25℃まで低下させた。仮に鋳型DNAとビオチンプローブの配列が一致すれば、その産物はビオチンとDIGのダブルラベル化体となる。それに対して鋳型DNAとビオチンプローブの配列が一致しない場合は、ダブルラベル化PCR産物は得られない。次にイムノクロマトグラフィーで結果を視覚化する。つまり得られた反応物を第一化学薬品製DNA検出ストリップにスポットし、展開した。DNA検出ストリップには、金コロイドラベルされた抗DIG抗体が塗布されたパッドとストレプトアビジンが塗布されたラインが存在し、PCR産物はサンプルパッド上に滴下され緩衝液により展開される。PCR産物がダブルラベル化されていればストレプトアビジンにビオチンがトラップされ、さらに金コロイドラベル抗DIG抗体が凝集し、バンドを形成する。つまりストリップを2本用意し、一方には野生型プローブを用いた時の反応液を、もう一方には変異型プローブを用いた時の反応液をアプライすることによって遺伝子型を視覚的に判別することができる。

(倫理面への配慮)

今回の研究プロトコールは本邦における「ヒトゲノム・遺伝子解析研究に関する倫理指針」を遵守し、東北薬科大学・大学院倫理委員会および東北大学医学部倫理委員会に申請・承認された同名課題「薬剤反応性遺伝子の多型性が薬効及び薬物動態に与える影響に関する研究」に従って実施された。

C. 研究結果

TPMT*3C(719A>G)検出において、従来のロシュ製のDNA検出ストリップを用いた場合、野生型検出ビオチンプローブ及び競合プローブは18mer、変異型検出ビオチンプローブ及び競合プローブは17merの合成オリゴヌクレオチドを用いていた。今回、第一化学薬品製のストリップを用いた場合でも、これらの鎖長プローブで最も感度良く、特異的に検出できることが判明した。同様に、CYP2C19 (CYP2C19*2(681G>A) 及び CYP2C19*3(636G>A))遺伝子多型診断系においても、ロシュ製ストリップで決定したプローブ鎖長の組合せが最も感度良く、正確に遺伝子診断できた。

さらにTPMT*3C(719A>G)遺伝子多型検出

系においては、DNA源として末梢血以外にも唾液で十分な遺伝子診断が可能であった。

D. 考察

従来用いていたロシュ製のDNA検出ストリップは、現在発売中止となり、入手することは困難である。そこで、第一化学薬品製の DNA 検出ストリップに変更したが、これまでのプローブ条件では最高のパフォーマンスが得られているか否かは不明であり、診断系のブラッシュアップが必要であった。今回、TPMT*3C、CYP2C19*2 及び CYP2C19*3 の遺伝子多型診断においては、それらのプローブ鎖長がロシュ製ストリップで設定したものと同様である時に最も高感度で正確に遺伝子診断できることが判明した。昨年度に検討したミトコンドリアDNA や NAT2 の診断系では、プローブ鎖長を変更することで感度の向上が認められたが、今回のケースでは変更は必要がなかった。また、今回の検討により DNA 源として唾液の利用が有効であることが判明した。唾液は血液よりも試料採取時の侵襲性が低く、将来遺伝子診断を基盤としたテーラーメイド医療が進展する上で有用なDNA源と考えられた。

E. 結論

これまでに我々が構築してきた薬剤反応性に関与する遺伝子多型診断法であるCASSOH法のブラッシュアップを行い、対象としたTPMT*3C、CYP2C19*2及びCYP2C19*3の検出では、プローブ鎖長を以前ロシュ製ストリップで設定した鎖長と同様な場合に最も高い検出感度と正確性が得られた。また、TPMT*3C検出系では唾液由来DNAを用いることでも遺伝子診断が可能であった。

F. 健康危険情報

G. 研究発表

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2. 学会発表

なし

H. 知的財産権の出願・登録状況

特になし

厚生労働科学研究費補助金(創薬基盤推進研究事業)

分担研究報告書

日本人集団における有用な薬理遺伝的遺伝子多型情報の収集

分担研究者　佐々木　崇光　東北薬科大学助手

研究要旨

近年、ヒトゲノム解析の進展により薬剤反応性の一部が薬物代謝酵素や薬物トランスポーター、薬物受容体遺伝子などのSNPの影響を大きく受けることが明らかにされている。将来的には薬物投与前に薬物動態や薬効発現に関連する遺伝子のSNP診断を行い、患者個々に最適な投与薬剤の選択及び投与量の調整を行うことが可能になる。このようなテーラーメイド医療の展開には、薬剤反応性に影響を及ぼすと考えられる遺伝子をリストアップすることが重要である。特に遺伝子多型に人種差が報告されているチオプリンメチルトランスフェラーゼ(TPMT)、CYP4A22及びCYP2D6遺伝子に関しては、日本人集団に特徴的なSNPが存在するため、詳細な解析が必要とされる。今回、日本人DNA検体を用いてTPMT及びCYP4A22遺伝子のSNPスクリーニングを行った。また、日本人集団で報告されているCYP2D6バリアントアレル由来の酵素タンパク質をCOS7細胞に発現させ、それらの機能解析を行った。その結果、TPMT遺伝子上にアミノ酸変異を伴う新規SNPを発見した。また、CYP4A22に関しては多様なバリアントアレルが存在することが明らかになった。さらに、CYP2D6バリアント酵素の中で活性低下を示すSNPが特定できた。これらの情報は、テーラーメイド医療を推進する上で有益と考えられる。

A. 研究目的

近年、薬剤反応性遺伝子における一塩基多型(SNP)解析に基づき、医薬品に対するレスポンダー・ノンレスポンダーの識別や副作用予測を行うことで、患者個々に適した投与薬剤の用法・用量の選択が可能になると考えられている。現在国内外において、種々の薬剤反応性遺伝子のSNPと投与薬物の血中濃度・治療効果、副作用発現等の相関性に関する情報が積み上げられている。しかし、薬物療法に影響を与えるSNPを有する患者に対して、臨床レベルでその診断を行い、薬物の適正使用を行っている施設は非常に少ないと言える。この原因として、簡易迅速な遺伝子診断法がスタンダード化されていないことが理由の一つとして考えられる。今回、「オーダーメイド薬物療法のための革新的なベッドサイド遺伝子診断法の開発と応用」の中で、臨床的に有用な薬剤反応性遺伝子のSNP検出法を開発すべく、その候補遺伝子を選択した。

B. 研究方法

薬剤反応性に影響を及ぼすと考えられる遺伝子 TPMT 及び CYP4A22 に関して、約 200 人の日本人ボランティア検体を用いて、SNP スクリーニングを行った。つまり、各エキソンを特異的に増幅するプライマーセットをデザインし、PCR を行った。電気泳動により単一バンドを確認し、それらの PCR 産物を Denaturing HPLC に導入し、クロマトグラムの変化から SNP の有無をスクリーニングした。これにより SNP の存在が疑われた検体については、ダイレクトシークエンス法により詳細な塩基配列の確認を行った。

CYP2D6 バリアントタンパク質の発現は、CYP2D6 cDNA を挿入した発現ベクターを構築し、COS7 細胞にトランスフェクトした。次に細胞を回収し、常法に従いミクロソーム画分を調製した。SNP の導入にはサイトダイレクトミュータジェネシス法を用いた。今回、これまでに日本人集団から同定されている CYP2D6*1、*2、*10、*14、*18、*27、*36、*39、*47、*48、*49、*50、*51、*53、*54、*55 及び*57 のバリアント酵素を発現させ、基質としてデキストロメトルファン O-脱メチル化活性を指標として酵素機能変化の有無を調査した。

(倫理面への配慮)

今回の研究プロトコールは本邦における「ヒトゲノム・遺伝子解析研究に関する倫理指針」を遵守し、東北薬科大学・大学院倫理委員会および東北大学医学部倫理委員会に申請・承認された同名課題「薬剤反応性遺伝子の多型性が薬効及び薬物動態に与える影響に関する研究」に従って実施された。

C. 研究結果

日本人DNA200検体中から、TPMTのエキソン3上にアミノ酸変異を伴う106G>A(Gly36Ser)を同定した。また、既知SNP(719A>G、Tyr240Cys; TPMT*3C)も同定された。これらSNPのアレル頻度はそれぞれ0.003及び0.010であった。これに加え、アミノ酸変異を伴わないSNPをそれぞれ2種類ずつ確認した。

日本人DNA191検体中から、CYP4A22のエキソン上に13箇所のSNPを同定し、さらにハプロタイプ解析により、21種バリアントアレルを特定した。これらのバリアントアレルは国際ヒトCYP命名委員会により、CYP4A22*1～*15と命名された。

野生型及びバリアント型CYP2D6を発現させたCOS7細胞から調製したミクロソーム画分50 μgを用いてデキストロメトルファンO-脱メチル化活性を測定した結果、野生型CYP2D6と比較して、CYP2D6.2、CYP2D6.27、CYP2D6.49、CYP2D6.50及びCYP2D6.55はデキストロメトルファン O-脱メチル化活性が有意に低下した。また、CYP2D6.10、CYP2D6.14、CYP2D6.18、CYP2D6.36 、CYP2D6.47 、CYP2D6.51 、CYP2D6.54及びCYP2D6.57においては、検出限界以下の活性であった。

D. 考察

TPMT遺伝子上に同定されたエキソン3の106G>Aにより生ずるGly36Ser変異は、これまでの報告により、酵素活性の低下が明らかなTPMT*13(83A>T、Glu28Val)や*17(124C>G、

Gln42Glu)と近傍の位置に存在する。また、メチル基供与体であるS-アデノシルメチオニンの結合部位も近いことから、TPMTの酵素学的諸性質に影響を及ぼす可能性が示唆された。

CYP4A22に関しては、これまでに130番目のグリシンがセリンに置換されると酵素活性が欠損することが報告されている。今回、同定したアレルの中ではエキソン4のSNP（4628C>A (Gly130Ser)）によりCYP4A22*4、*10、*12A、*12B、*13A、*13B、*14及び*15において、130番目がセリンに置き換わる。したがって、これらのアレル由来のCYP4A22タンパクは酵素活性が欠損することが示唆された。

CYP2D6に関して、日本人において最も高頻度のバリアントアレルであるCYP2D6*10を有するヒトにおいては、デキストロメトルファンO -脱メチル化活性がCYP2D6*1に比べ有意に低いことが知られている。またin vitroの研究においてCYP2D6.10の2つの変異のうちP34S置換が主な原因となってデキストロメトルファン O -脱メチル化活性が低下することが明らかとなっており、今回P34S置換を有するすべてのバリアント酵素CYP2D6（CYP2D6.10、CYP2D6.14、CYP2D6.36 、 CYP2D6.47 、 CYP2D6.49 、CYP2D6.54及びCYP2D6.57）においても活性の低下が認められた。

E. 結論

今回、遺伝子多型診断によるテーラーメイド医療の実現化を図るべく、日本人集団に特徴的な薬物代謝酵素遺伝子多型の解析を行った。その結果、薬物療法で遺伝子多型の影響を受けると考えられるTPMT遺伝子上にアミノ酸変異を伴う新規SNPを発見した。また、CYP4A22に関しては多様なバリアントアレルが存在することが明らかになった。さらに、CYP2D6バリアント酵素の中で活性低下を示すSNPが特定できた。

F. 健康危険情報

G. 研究発表

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H. 知的財産権の出願・登録状況

特になし

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

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雑誌

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Hiratsuka M, Ebisawa A, Sakuyama K, Matsubara Y, Kure S, Soya Y, Konno Y, Sasaki T, Kishiba A, Mizugaki M.	Competitive allele-specific short oligonucleotide hybridization (CASSOH) with enzyme-linked immunosorbent assay (ELISA) for the detection of pharmacogenetic single nucleotide polymorphisms (SNPs)	J Biochem Biophys Methods	67	87-94	2006
Niihori T, Aoki Y, Narumi Y, Neri G, Cave H, Verloses A, Okamoto N, Hennekam RC, Gillessen-Kaesbach G, Wieczorek D, Kavamura MI, Kurosawa K, Ohashi H, Wilson L, Heron D, Bonneau D, Corona G, Kaname T, Naritomi K, Baumann C, Matsumoto N, Kato K, Kure S, Matsubara Y.	Germline KRAS and BRAF mutations in cardio-facio-cutaneous syndrome.	Nat Genet	38	294-296	2006

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Ⅳ. 研究成果の刊行物・別刷

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Review

Genetic testing for pharmacogenetics and its clinical application in drug therapy

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Abstract

There is wide individual variation in drug responses and adverse effects. As the main causes of the variation in drug responses, attention has focused on the genetic polymorphisms that encode metabolic enzymes regulating pharmacodynamics and receptors modulating the affinity with the responsive sites. Tailor-made drug therapy analyzes genetic polymorphisms involved in drug responses before drug administration and selects drugs and doses suitable for the individual genetic background. Establishment of tailor-made drug therapy is expected to contribute to medical economy by avoiding wasteful drug administration. To promote such medical practice, it is necessary to use simple genetic testing that is clinically convenient. Currently, genetic testing using real-time PCR has been frequently employed at laboratories with its clinical application anticipated. As to the many genes involved in drug responses, to date, the application of patient genetic information to tailor-made drug therapy has been achieved at the practical level. Information on pharmacogenetics will be a critical factor in medical practice in the near future.
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Keywords: Genetic testing; Genetic polymorphism; Pharmacogenetics; Real-time PCR; Single nucleotide polymorphism; Tailor-made drug therapy

Contents

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1. Introduction

There is wide individual variation in the pharmacodynamics of the administered drugs, effectiveness, and the appearance rates of adverse effects, and they are regulated by functions of the liver, kidneys, and heart, age, gender, circadian rhythm, diet, concomitantly administered drugs, and healthy foods. Recently, genetic polymorphism has drawn attention as one of such factors [1]. In particular, a single nucleotide polymorphism (SNP) of DNA is thought to produce a variation in drug responses and has become a representative research target in pharmacogenetics. In some cases, a difference in only one nucleotide results in a several- to ten-fold increase in maximum drug concentrations in the blood and frequent incidences of unexpected critical adverse effects. Today, it is anticipated that such genome information will be used to provide safer and more efficient tailor-made medicine for patients. If patient genome information is investigated before drug administration and appropriate drugs, doses, and administration timing can be predicted, a minimum drug dose will yield the maximum effect with the minimum side effects. If such medical practice is available, low efficiency in selecting drugs on a trial and error basis and drug administration as symptomatic therapy in cases of the emergence of side effects are expected to greatly change, which will contribute to medical economy. To date, polymorphisms of genes encoding drug metabolism enzymes, drug transporters, and drug receptors, which are involved in drug responses, have been reported and in some of them the association between pharmacodynamics and drug efficacy has been clarified [1,2].

In the future the expansion of tailor-made medicine using genome information, the development of simple and rapid genetic testing will be critical. Especially in the case of outpatients, unless results are returned within some tens of minutes to a few hours after the order of genetic testing, genome information cannot be used for prescription on the same day. Recently, new systems have been developed to detect genetic polymorphisms involved in drug responses in about 30 min to 2 hours by using real-time PCR.

In this review, we summarize the recent information on genetic polymorphisms involved in drug responses and demonstrate recent genetic testing including real-time PCR, the clinical application of tailor-made drug therapy, and the impact on medical economy.

2. Target genes of pharmacogenetics

2.1. P450 enzymes

A number of enzymes are involved in drug metabolism, and cytochrome P450 (CYP, P450) is regarded as the most important enzyme involved in drug metabolism, because many drugs are the substrate for the enzyme. P450 forms a superfamily consisting of a variety of molecules, and almost 20 kinds of enzymes involved in drug metabolism have been confirmed in humans (http://drnelson.utmem.edu/CytochromeP450.html). Of them, CYP3A4, CYP2D6, CYP2C9, CYP2C19, CYP2B6, and CYP1A2 play the most critical role in drug metabolism, and account for more than 90% of drugs metabolized by P450 [1,3]. P450 enzymes involved in the metabolism of the drugs currently available in medical practice are shown in Table 1. These molecules have proven genetic polymorphisms (Table 2), and a number of reports have shown the association between drug responses and genetic polymorphisms, in particular, as to CYP2D6, CYP2C9, and CYP2C19. Updated information can be found on the Human CYPallele Nomenclature Website (http://www.imm.ki.se/cypalleles). Phenotypes of P450 are divided into the extensive metabolizer (EM) that shows regular metabolic capacity and the poor metabolizer (PM) that shows low metabolic capacity. The PM phenotype carrying gene alterations on both alleles is inherited in an autosomal recessive manner. In addition, the categorization includes the intermediate metabolizer (IM) that shows the metabolic capacity between the PM and the EM, and the ultra-rapid metabolizer (UM) that shows higher metabolic capacity than the EM.

2.1.1. CYP2D6

In cases with CYP2D6, 5–10% of Caucasians are the PMs that have little enzymatic activity [4]. The frequency of the PM has racial diversity and the frequency of the CYP2D6 PM is less than 1% in Mongolian-origin races including Japanese [5,6]. Metabolism is delayed in a variety of drugs in the PM, and even the same drug dose is more likely to cause side effects, because plasma concentrations of the drug last longer at higher than normal levels. For example, in cases with the CYP2D6 PM, tricyclic antidepressants cause arrhythmia and thirstiness at higher rates [7]. To date, almost 50 genetic polymorphisms for CYP2D6

Table 1
Substrates of drug-metabolizing enzymes

Enzymes		Substrates
CYP1A2	Antidepressants	amitriptyline, clomipramine, fluvoxamine, imipramine
	Miscellaneous	clozapine, cyclobenzaprine, estradiol, haloperidol, mexiletine, naproxen, olanzapine, ondansetron, propranololriluzole, ropivacaine, tacrine, theophylline, verapamil, *R*-warfarin, zileuton, zolmitriptan
CYP2B6	Chemotherapeutic agents	cyclophosphamide, ifosfamide
	Miscellaneous	alfentanil, bupropion, efavirenz, ketamine, methadone, nevirapine, propofol, tamoxifen,
CYP2C9	Anticoagulants	*S*-warfarin
	Sulfonylureas	glibenclamide, glimepiride, glipizide, tolbutamide,
	NSAIDs	celecoxib, diclofenac, ibuprofen, mefenamic acid, meloxicam, naproxen, piroxicam, suprofen
	Antidepressants	amitriptyline, fluoxetine, imipramine
	Antihypertensives	irbesartan, losartan
	Miscellaneous	fluvastatin, nateglinide, phenytoin, rosiglitazone, tamoxifen, torsemide
CYP2C19	Benzodiazepines	diazepam
	Antidepressants	amitriptyline, clomipramine, imipramine
	Proton pump inhibitors	lansoprazole, omeprazole, pantoprazole, rabeprazole
	Miscellaneous	carisoprodol, citalopram, cyclophosphamide, hexobarbital, indomethacin, *S*-mephenytoin, mephobarbital, nelfinavir, nilutamide, phenobarbitone, phenytoin, primidone, progesterone, proguanil, propranolol, teniposide
CYP2D6	Antiarrhythmics	amiodarone, aprindine, encainide, finexiletine, idocaine, lecainide, lsparteine, propafenone, *N*-propylajmaline
	Opiates	codeine, dextromethorphan, dihydrocodeine, ethlmorphine, hydrocodone, norcodeine, oxycodone, tramadol
	Antihypertensives	alprenolol, carvedilol, bufuralol, bunitrolol, bupranolol, debrisoquine, guanoxan, indoramin, *S*-metoprolol, propranolol, timolol
	Antidepressants	amiflamine, amitriptyline, brofaromine, citalopram, clomipramine, desipramine, desmethylcitalopram, fluoxetine, fluvoxamine, imipramine, maprotiline, minaprine, moclobemide, nortriptyline, paroxetine, tomoxetine, trimipramine, venlafaxine
	Antipsychotics	chlorpromazine, clozapine, haloperidol, perphenazine, risperidone, sertindole, thioridazine, zuclopentixol
	Miscellaneous	cinnarizine, dolansetron, methoxyamphetamine, methoxyphenamine, metoclopramide, nicergoline, ondansetron, phenformin, promethazine, tamoxifen, tolterodine, tropisetron
CYP3A4	Immune modulators	cyclosporine, tacrolimus
	Benzodiazepines	alprazolam, diazepam, midazolam, triazolam
	Ca channel blockers	amlodipine, diltiazem, felodipine, lercanidipine, nefedipine, nisoldipine, nitrendipine, verapamil
	Chemotherapeutic agents	busulfan, docetaxel, etoposide, irinotecan, paclitaxel, tamoxifen, vinblastine, vincristine
	HMG-CoA reductase inhibitors	atorvastatin, cerivastatin, lovastatin, simvastatin
	Estrogens, corticosteroids	estradiol, hydrocortisone, progesterone, testosterone
	Macrolide antibiotics	clarithromycin, erythromycin
	Protease inhibitors	indinavir, nelfinavir, ritonavir, saquinavir
	Antihistamines	astemizole, chlorpheniramin, terfenadine
	Miscellaneous	buspirone, cisapride, cilostazol, cocaine, dapsone, dextromethorphan, domperidone, fentanyl, finasteride, imatinib, lidocaine, methadone, nateglinide, pimozide, ondansetron, quinine, salmeterol, sidenafil, sirolimus, trazodone, zaleplon, zolpidem
TPMT	Chemotherapeutic agents	mercaptopurine, thioguanine
	Immune modulators	azathiprine
DPD	Chemotherapeutic agents	carmofur, doxifluridine, 5-fluorourasil, tegafur
NAT2		isoniazid, hydralazine, procainamide, sulfamethoxazole
UGT1A1		bilirubin, irinotecan
COMT		levodopa

have been reported (http://www.imm.ki.se/cypalleles). Of them, CYP2D6*3, CYP2D6*4, and CYP2D6*5 are the major genetic polymorphisms involved in the PM of Caucasians [8]. More than 90% of the PMs in Caucasians are ascribable to these three genetic polymorphisms. Moreover, the existence of CYP2D6*10, a genetic polymorphism of the IM that shows lower CYP2D6 metabolic capacity but not as low as the PM, has been identified [9]. This genetic polymorphism is markedly prevalent in Japanese and Chinese.

2.1.2. CYP2C9

CYP2C9 is involved in the metabolism of an antiepileptic agent phenytoin and an anticoagulant warfarin. To date, 12 CYP2C9 variant alleles have been reported (http://www.imm.ki.se/cypalleles). Of these, a decrease in activity was confirmed in cases with CYP2C9*3 by the expression system using COS cells and yeast and in vivo tests on the normal volunteers and patients whose genetic polymorphisms were known [10,11]. For example, in cases with phenytoin, oral clearance decreased to one quarter in the

Table 2
Pharmacogenetics of drug-metabolizing enzymes (P450)

Gene	Genotypes	Major allelic variants	Phenotypes: Frequency	
			Caucasian	Asian (Japanese)
CYP1A1	*1–*11			
CYP1A2	*1–*14	*1F, *2, *3, *4, *5, *6		
CYP1B1	*1–*26			
CYP2A6	*1–*17	*2, *4, *7, *9, *10		
CYP2A13	*1–*9			
CYP2B6	*1–*15			
CYP2C8	*1–*5			
CYP2C9	*1–*13	*3	EM: 97% PM: 3%	
CYP2C19	*1–*16	*2, *3	EM: 94–98% PM: 2–6%	EM: 80% PM: 20%
CYP2D6	*1–*51	*2×N, *3, *4, *5, *6, *10, *17	EM: 90–97%	EM: 99%
			PM: 3–10%	PM: 1%
CYP2E1	*1–*7	*2, *3, *4		
CYP2J2	*1–*7			
CYP2R1	*1–*2			
CYP2S1	*1–*3			
CYP3A4	*1–*19	*4, *5, *6		
CYP3A5	*1–*10	*3, *6		
CYP3A7	*1			
CYP3A43	*1–*3			
CYP4B1	*1–*7			

A description of the alleles can be found on the human cytochrome P450 allele nomenclature committee home page (http://www.imm.ki.se/CYPalleles/).
EM=extensive metabolizer; IM=intermediate metabolizer; PM=poor metabolizer.

subjects with homozygous polymorphism for CYP2C9*3 [12]. Oral clearance for (S)-warfarin decreased to less than half in the subjects with heterozygous polymorphism for CYP2C9*3 (CYP2C9*1/*3) and to less than 10% in the patients with homozygous polymorphism for CYP2C9*3 [13].

2.1.3. CYP2C19

CYP2C19 is an enzyme involved in the hydroxylation of the S form of an anti-epileptic agent mephenytoin, and also in the metabolism of a series of proton pump inhibitors such as omeprazole. The PM for CYP2C19 is found in about 20% of Japanese and about 3% of Caucasians [14]. To date, 15 variant alleles responsible for the PM for CYP2C19 have been identified (http://www.imm.ki.se/cypalleles), and almost all PMs in Japanese are ascribable to the two genetic polymorphisms CYP2C19*2 and CYP2C19*3. Omeprazole concentrations in the blood after oral intake of omeprazole were significantly different in each genetic polymorphism for CYP2C19, and the concentration in the blood 10 h after the intake in the PM was comparable to the peak concentration in the EM [15]. When the area under the plasma concentration–time curve was compared, the PM was reported to be different from the EM by about 13 times [16].

2.2. Non-P450 enzymes

2.2.1. Dihydropyrimidine dehydrogenase (DPYD)

Dihydropyrimidine dehydrogenase (DPD) is a rate-limiting enzyme for the metabolism of an anticancer drug 5-FU, and more than 85% of administered 5-FU is metabolized by DPD. The gene encoding DPD is called DPYD, and 13 genetic polymorphisms have been reported [17,18]. In particular, the genetic polymorphism that decreases DPD activity has been reported to be DPYD*2 with a polymorphism at the splicing recognition site [19]. Administration of 5-FU to the patients with decreased DPD activity increases the adverse events such as leukocytopenia, stomatitis, diarrhea, nausea and vomiting, and cerebellum disorder [20].

2.2.2. Thiopurine S-methyltransferase (TPMT)

Thiopurine *S*-methyltransferase (TPMT) is involved in the detoxification and metabolism of an anti-leukemia drug 6-mercaptoprine (6-MP) and an immunosuppressant azathioprine. In Caucasian infant patients with acute myeloid leukemia, those who carried TPMT*2, TPMT*3A, TPMT*3B, or TPMT*3C showed significantly higher concentrations of the 6-MP metabolite in the red blood cells. In addition, a dose reduction or termination of the administration was reported to be necessary in all patients due to the adverse effects such as myelosuppression [21].

2.2.3. N-acetyltransferase 2 (NAT2)

An individual variation in *N*-acetylation activity of an anti-tuberculosis drug isoniazid has been reported to be ascribable to the genetic polymorphism for *N*-acetyltransferase 2 (NAT2). NAT2 is categorized into the rapid acetylator (RA), the intermediate acetylator (IA), and the slow acetylator (SA) according to the acetylation activity. The frequency of the SA is about 50% in Caucasians and about 10% in Japanese [22,23]. To date, more than 10 variant alleles have been identified (http://www.louisville.edu/medschool/pharmacology/NAT.html), and three genetic polymorphisms NAT2*5, NAT2*6, and NAT2*7, but not NAT2*4 (wild type alleles), are responsible for almost all SAs in Japanese [23]. Drug-induced hepatitis by isoniazid occurs more than twice in the SA for NAT2 than in the RA [24,25].

2.2.4. Other non-P450 enzymes

Other than the above-mentioned enzymes for drug metabolism, genetic polymorphisms for UDP-glucuronic acid transferase (UGT) 1A1 [26], glutathione-*S*-transferase (GST) [27], and thymidylic acid synthase (TS) [28] are thought to be responsible for the individual variation in drug responses. The relation between these genes and drug responses is shown in Table 3.

Table 3
Pharmacogenetics of drug-metabolizing enzymes (non-P450)

Drug-metabolizing enzymes	Gene	Genotypes	Major allelic variants	Phenotypes: Frequency	
				Caucasian	Asian (Japanese)
Thiopurine *S*-methyltransferase	TPMT	*1–*15	*2, *3	EM: 73–89% IM: 11–27% PM: 0–4%	EM: 97% IM: 3%
Dihydropyrimidine dehydrogenase	DPYD	*1–*12	*2	EM: 97% PM: 3%	
N-acetyltransferase 2	NAT2	*4–*19	*5, *6, *7, *14	EM: 25% IM: 25% PM: 50%	EM: 45% IM: 45% PM: 10%
UDP-glucuronosyltransferase 1A1	UGT1A1	*1–*64	*6, *7, *27, *28, *29	EM: 90% PM: 10%	EM: 99% PM: 1%
Catechol O-methyltransferase	COMT		Val158Met	EM: 75% PM: 25%	
Glutathione *S*-transferase M1	GSTM1		null		
Glutathione *S*-transferase M3	GSTM3		*A, *B		
Glutathione *S*-transferase-P1	GSTP1		Ile105Val		
Glutathione *S*-transferase-T1	GSTT1		null		

EM = extensive metabolizer; IM = intermediate metabolizer; PM = poor metabolizer.

2.3. Non-enzymes

Recently, genetic polymorphisms, other than those for enzymes for drug metabolism, have drawn attention as important factors for drug responses. They include those for drug transporters, receptors, transport protein, and mitochondria DNA (mtDNA) (Table 4). Especially, the focus has been on genetic polymorphisms for receptors that can be the molecular target for drugs. An anti-lung cancer drug gefitinib binds the site for epidermal growth factor receptor-tyrosine kinase (EGFR-TK) and blocks the signal transduction for cancer proliferation. An individual variation in the effect of gefitinib was reported [29–31], and good response was observed in the patients with a polymorphism in the EGFR-TK domain. On the other hand, gefitinib was not effective in the patients without polymorphisms in the EGFR-TK domain.

3. Genotyping methods

Recent progress in genetic testing technologies is remarkable, and they are applied for the analysis of genetic mutations and polymorphisms that regulate disease vulnerability and drug responses. Classically, the PCR-RFLP analysis and the allele-specific amplification method had been used for the detection of SNP [32]. However, these methods require rather troublesome maneuvers such as electrophoresis after PCR and staining with ethidium bromide. A few years ago a new method was developed to cover the shortcomings of these genetic tests. The method is called real-time PCR because it uses a fluorescent probe or dye in the PCR reaction to detect DNA amounts and characteristic base compositions over time [33,34]. These methods allow the detection of SNP within about 40 min to 2 h of DNA extraction. For real-time PCR, the TaqMan probe and Hybridization probe methods are used in a number of institutes to detect various SNPs (Table 5).

3.1. Real-time PCR

3.1.1. TaqMan probe

The TaqMan probe method employs 5′-exonuclease activity of Taq DNA polymerase for PCR. The TaqMan probe consists of an oligo-nucleotide of about 20–30-mer and labeled with a reporter fluorescent dye at the 5′-terminus and a quencher fluorescent dye at the 3′-terminus. The two fluorescent dyes on the TaqMan probe are in a state of decreased fluorescent intensity due to fluorescence resonance energy transfer (FRET) phenomenon when they are close in physical distance. However, the 5′-exonuclease of Taq DNA polymerase cleaves the binding of the fluorescent dye along the extension reaction. The fluorescent dye is released from the influence of the quencher and fluoresces. In general, the TaqMan probe is designed to be at the center of SNP.

Recently, we developed a new real-time PCR method using allele-specific amplification [38]. We designed PCR primers at the site of SNP so that not only the TaqMan probe, but also less expensive SYBR Green I is available [38,54]. ABI PRISM7000, 7300, 7500, PRISM7700, 7900Fast (Applied Biosystems), LightCycler (Roche), Mx4000, Mx3000P (STRATAGENE), and Smart Cycler II (TaKaRa) can be used for detection.

3.1.2. Hybridization probe

The Hybridization Probe method allows real-time detection of PCR products and SNP using two fluorescent probes. Firstly, PCR primers are designed to amplify the target sequence including SNP. Secondly, a probe labeled with fluorescein at the 3′-terminus is designed so that it

Table 4
Genes associated with altered drug effect

Name	Gene	Drug
MDR1, *P*-glycoprotein	ABCB1	digoxin
Angiotensin converting enzyme	ACE	ACE inhibitors
β-1 adrenergic receptor	ADRB1	β adrenergic receptor antagonists
β-2 adrenergic receptor	ADRB2	albuterol, salbutamol
Angiotensinogen	AGT	antihypertensive drugs
Angiotensin-II receptor type1	AGTR1	angiotensin-II receptor antagonists
Arachidonate 5-lipoxygenase	ALOX5	5-lipoxygenase inhibtors, leukotriene receptor antagonists
Bradykinin receptor B2	BDKRB2	ACE inhibitors
Cholesteryl ester transfer protein	CETP	pravastatin, atorvastatin
Dopamine D2 receptor	DRD2	haloperidol, nemonapride
Dopamine D3 receptor	DRD3	clozapine
Dopamine D4 receptor	DRD4	neuroleptics
Epidermal growth factor receptor	EGFR	gefitinib, erlotinib
Guanine nucleotide-binding protein, B3 subunit	GNB3	antidepressants
N-methyl-D-aspartate receptor 2B subunit	GRIN2B	clozapine
5-hydroxytryptamine receptor 2A	HTR2A	antipsychotic drugs
Inositol polyphosphate 1-phosphatase	INPP1	lithium
Inosine triphosphate pyrophosphatase	ITPA	azathioprine
Hepatic lipase	LIPC	statins
Leukotriene C4 synthase	LTC4S	zafirlukast, pranlukast
Mitochondrial DNA	mtDNA	aminoglycoside antibiotics
5, 10-Methylenetetrahydrofolate reductase	MTHFR	methotrexate, 5-fluorourasil
Organic anion transporting polypeptide-C	OATP-C	pravastatin
Peroxisome-proliferator activated receptors alpha	PPARA	fenofibrate
Dopamine transporter	SLC6A3	methylphenidate
Serotonin transporter	SLC6A4	fluoxetine, paroxetine
Tryptophan hydroxylase 1	TPH1	antidepressants
Thymidylate synthase	TYMS	5-fluorourasil

can hybridize the sequence in the PCR product amplified with these primers. At the close site, another probe labeled with LCRed640 at the 5′-terminus and phosphate at the 3′-terminus is designed. They are designed so that SNP is located at the center of either one of these probes. SNP is detected by the analysis of the melting curve. In other words, temperature is raised slowly after PCR with fluorescent signals monitored. At a certain temperature, a probe with lower Tm dissociates and fluorescein is separated from LCRed640, and fluorescent intensity drastically plummets. In other words, a mismatch between the template and the probe allows dissociation more easily than the completely matched sequence, and as a result the presence or absence of SNP is detected. LightCycler (Roche) is required for the detection. Roche has already started the sales of mutation detection kits for CYP2C9 (*2 and *3), CYP2C19 (*2 and *3), and NAT2 (*5, *6, *7 and *14) on a commercial basis.

3.1.3. Other real-time PCR

HyBeacon Probe [55] and a real-time PCR method with a 3′-locked nucleic acid primer [56] have been reported for the detection of SNP. In addition, High-Resolution Melting Assay with a fluorescent dye LCGreen I [57,58], which can be incorporated into double-strand DNA in a saturated manner, has been developed to cover the shortcomings of SYBR Green I that was previously used. Other than PCR, for the amplification methods at the fixed temperature, loop-mediated isothermal amplification (LAMP) [59] and isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN) (http://www.takara.co.jp/english/index.htm) have been developed. These gene amplification methods do not require an expensive thermalcycler and have the advantage that a less expensive block incubator can be used. However, designing primers is more troublesome than PCR and requires special software.

3.2. Other methods

As other genetic tests, Denaturing High Performance Liquid Chromatography (DHPLC) [60], Pyrosequencing [61], Invader assay [62,63], Luminex assay [64], Mass Array using Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) [65], SNPstream [66], DNA Chip [67], Nano Chip [68], and eSensor chip [64] have been widely used in laboratories. Regarding DNA Chip, Roche has started sales of AmpliChip CYP450 on a commercial basis, which can detect CYP2D6 polymorphisms (http://www.roche-diagnostics.com/products_services/amplichip_cyp450.html).

3.3. Point-of-care testing

Currently, the TaqMan Assay, melting curve analysis, and direct sequencing are genetic testing technologies generally used in the laboratories. On the other hand, Pyrosequencing, TOF-MS, and DNA Chip, which require relatively expensive specific equipment and special techniques, are used in entrusted analysis institutes and genome centers. For the application of tailor-made drug therapy to clinical settings, point-of-care testing that allows easy genetic testing at the patient bedside and the clinic will be necessary in the future. We recently developed point-of-care genetic testing using immunochromatography [69,70]. In this method, the reaction after PCR is dropped on a strip and visual judgment of the SNP becomes possible by the appearance of a purple line on the strip when SNP is present. Detection requires 10 min after PCR and the method allows a simple and rapid genetic testing.

Table 5
Examples of pharmacogenetic markers detected by real-time PCR assay (TaqMan assay and Hybridization probe assay)

TaqMan assay	Hybridization probe assay
ADRB2 (46A>G) [35]	ADRB2 (46A>G) [43]
ADRB2 (79C>G) [35]	ADRB2 (79C>G) [43]
CETP (270C>T, B2 allele) [36]	CETP (270C>T, B2 allele) [43]
CYP2B6 (516G>T, *6,*7, *9 or *13 allele) [37]	CYP1A1 (4889A>G) [44]
CYP2C9 (416C>T, *2 allele) [38]	CYP1B1 (432C>G, *2 allele) [45]
CYP2C9 (1061A>C, *3 allele) [38]	CYP2C9 (416C>T, *2 allele) (www.roche-applied-science.com)
CYP2C19 (681G>A, *2 allele) [38]	CYP2C9 (1061A>C, *3 allele) (www.roche-applied-science.com)
CYP2C19 (636 G>A, *3 allele) [38]	CYP2C19 (681G>A, *2 allele) (www.roche-applied-science.com)
CYP2D6 (gene duplication, *2 × 2 allele) [39]	CYP2C19 (636 G>A, *3 allele) (www.roche-applied-science.com)
CYP2D6 (1846G>A, *4 allele) [38]	CYP2D6 (-1584 C>G, *2 allele) [46]
CYP2D6 (gene deletion, *5 allele) [39]	CYP2D6 (gene duplication, *2 × 2 allele) [46]
CYP2D6 (100C>T, *10 allele) [38]	CYP2D6 (gene deletion, *5 allele) [46]
CYP2D6 (1758G>A, *14 allele) [38]	CYP2D6 (31 G>A, *35 allele) [46]
CYP2D6 (4125–4133insGTGCCCACT, *18 allele) [38]	GSTM1 (gene deletion) [47]
CYP2D6 (2573insC, *21 allele) [38]	GSTT1 (gene deletion) [47]
CYP3A5 (6981A>G, *3 allele) [37,40]	GSTP1 (A>G, Ile105Val allele) [47]
CYP3A5 (14685A>G, *6 allele) [37]	HTR2A (102T>C) [43]
LTC4S (-444A>C) [41]	INPP1 (973C>A) [43]
MDR1 (-129T>C) [42]	MDR1 (3435C>T) [48]
MDR1 (325G>A) [42]	mtDNA (1555A>G) [43]
MDR1 (2677G>T/A) [42]	MTHFR (677C>T) [49,50]
MDR1 (3435C>T) [42]	NAT2 (341T>C, *5 allele) [51]
NAT2 (341T>C, *5 allele) [38]	NAT2 (481C>T, *5 allele) [52]
NAT2 (481C>T, *5 allele) [38]	NAT2 (590G>A, *6 allele) [52]
NAT2 (590G>A, *6 allele) [38]	NAT2 (857G>A, *7 allele) [52]
NAT2 (857G>A, *7 allele) [38]	NAT2 (191G>A, 14 allele) (www.roche-applied-science.com)
TPMT (719A>G, *3C) [38]	TPMT (238G>C, *2 allele) [53]
	TPMT (460G>A, 719A>G, *3A) [53]
	TPMT (460G>A, *3B) [53]
	TPMT (719A>G, *3C) [53]
	TPMT (292G>T, 460G>A, 719A>G, *3D) [53]
	TPMT (intron9/exon10 splice junction G>A, *4 allele) [53]
	TPMT (146T>C, *5 allele) [53]
	TPMT (539A>T, *6 allele) [53]
	TPMT (681T>G, *7 allele) [53]

4. Clinical application

There are a number of reports on the association of genetic polymorphisms involved in drug responses with pharmacodynamics, efficacy, and adverse effects, but few cases have been clinically applied. In particular, evidence that supports clinical application has been accumulated for NAT2, CYP2C19, TPMT, and mtDNA A1555G.

4.1. NAT2 genotyping for isoniazid treatment

Since NAT2 is involved in the acetylation of isoniazid, sulphamethazine, and procaine amide, blood concentrations of these drugs are expected to increase and result in higher incidences of adverse events in the SA for NAT2. Since rifampicin, often concomitantly administered with isoniazid for tuberculosis, induces an oxidative hydrolysis enzyme and the production of hydralazine, a toxic metabolite, it has been reported that the drug is likely to cause liver disorder in SA patients [24].

We conducted genetic testing for NAT2 by real-time PCR on 102 Japanese patients receiving isoniazid, without concomitant administration of rifampicin [71]. As a result, adverse events appeared in six patients in total and in 83.3% of the SA for NAT2. There were various adverse events such as nausea, vomiting, fever, visual disturbance, and peripheral nerve injury, and reduction or termination of isoniazid successfully decreased or eliminated adverse events in these patients. The adverse effects of isoniazid were not so critical, but genetic testing for NAT2 should be clinically applied so that the administration does not decrease the QOL of the patients.

4.2. CYP2C19 genotyping for omeprazole treatment

Proton pump inhibitors have a potent acid suppressive effect, and a combination with antibiotics is used to eradicate *Helicobacter pylori* (*H. pylori*) and treat ulcer in the upper gastrointestinal tract. Proton pump inhibitors are mainly metabolized by CYP2C19 and the pharmacodynamics and efficacy are influenced by the CYP2C19 polymorphism. Furuta et al. [72] found that the eradication rates of *H. pylori* with omeprazole 20 mg/day plus amoxicillin 2000 mg/day for 6 or 8 weeks were 28.6% in the EM, 60.0% in the IM, and 100.0% in the PM, and there was a significant difference among genetic polymorphisms. In the PM, the aforementioned doses of the two drugs, a proton pump inhibitor and amoxicillin, yielded a sufficient eradication rate, and the necessity to increase the dose of a proton pump inhibitor in the EM and the IM was verified clinically. Furthermore, CYP2C19 polymorphism affects treatment efficacy by the mainstream three-drug regimen. The eradication rates of *H. pylori* with omeprazole 40 mg/day or lansoprazole 60 mg/day, and amoxicillin 1500 mg/day and clarithromycin 600 mg/day for 1 week were 72.7% in the EM,