

Review

Pharmacogenomics-based clinical studies using a novel, fully automated genotyping system

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ABSTRACT: Clinical investigations into single nucleotide polymorphisms (SNPs) in drug metabolism have already been set out for clinical trials in subject groups classified as extensive metabolizers or poor metabolizers. In particular, the frequency of CYP2C19 in poor metabolizers within the Japanese population is relatively high, and genetic variations result in differences in kinetics and pharmacological action, e.g. clinical response to proton pump inhibitors which are mainly metabolized by 2C19 in the liver. We introduced a novel, fully automated genotyping system and used it in the genotyping of CYP2C19. The completed system is based on the analysis of a melting curve of probe DNA which is bound to the target SNP site using a fluorescence quenching probe. The system enables automated and multiple SNP-genotyping from sample preparation. This fully automated system of analysis can be adapted to clinical studies, e.g. classification of genes related to pharmacokinetics and target receptors by genetic variations.

Keywords: Pharmacogenomics, Automated genotyping system, Single nucleotide polymorphism, CYP2C19

1. Introduction

With the advent of a post-genome era, clinical studies associated with drug development are likely to change drastically. In particular, noticeable improvements are expected in phase-I clinical studies where healthy adults are enrolled as trial subjects. With the introduction of pharmacogenomics, evidence-based medications which are based on genome information will become available and result in improved safety and efficacy (1). Sekino Clinical Pharmacology Clinic (SCPC) has developed

into one of the leading facilities for Phase I clinical trials in Japan. In response to drug development in the post-genomic era, this Clinic is taking a proactive approach to the introduction of pharmacogenomics into clinical trials.

Since determination of the human genome sequence, two main streams of personalized medicine have appeared in the post-genomic era: one is genomic-based drug discovery and the other is individual single nucleotide polymorphism (SNP) typing. SNP is the most common form of DNA sequence variation occurring when a single nucleotide in the genome differs between members of the species, ethnicity, and individuals. Genotyping of SNPs is of great value to biomedical research and in developing personalized medicine because it can affect how humans respond to pathogens, chemicals, and drugs in particular (2-5).

Conventionally, gene-typing was performed using several large pieces of equipment that must be operated manually. In addition to relatively large space, it also required technical experts to properly operate the complicated system. Outsourcing of gene-typing is not a viable option because of ethical concerns relating to safety management of DNA and security of genomic information. Recently, this Clinic has implemented a novel, fully automated, easy-to-use, compact genotyping system in Pharmacogenomics (PGx)-based clinical studies, and this system could contribute to in-house analysis of SNPs by non-technical experts.

2. Background of PGx-related clinical trials and the current state of PGx-trials in Japan

Clinical trials are usually classified into three phases. Phase I trials are primarily concerned with assessing a drug's safety. This initial phase of testing in humans is done in a small number of healthy volunteer subjects. The study is mainly designed to determine what happens to the drug in the human body; in other words, it is a pharmacokinetics study. Phase II and III trials for patients continue to test the safety of the drug and begin to evaluate how well the new drug works, which are known as pharmacodynamics studies. In basic terms, pharmacokinetics is the study of what a body does to

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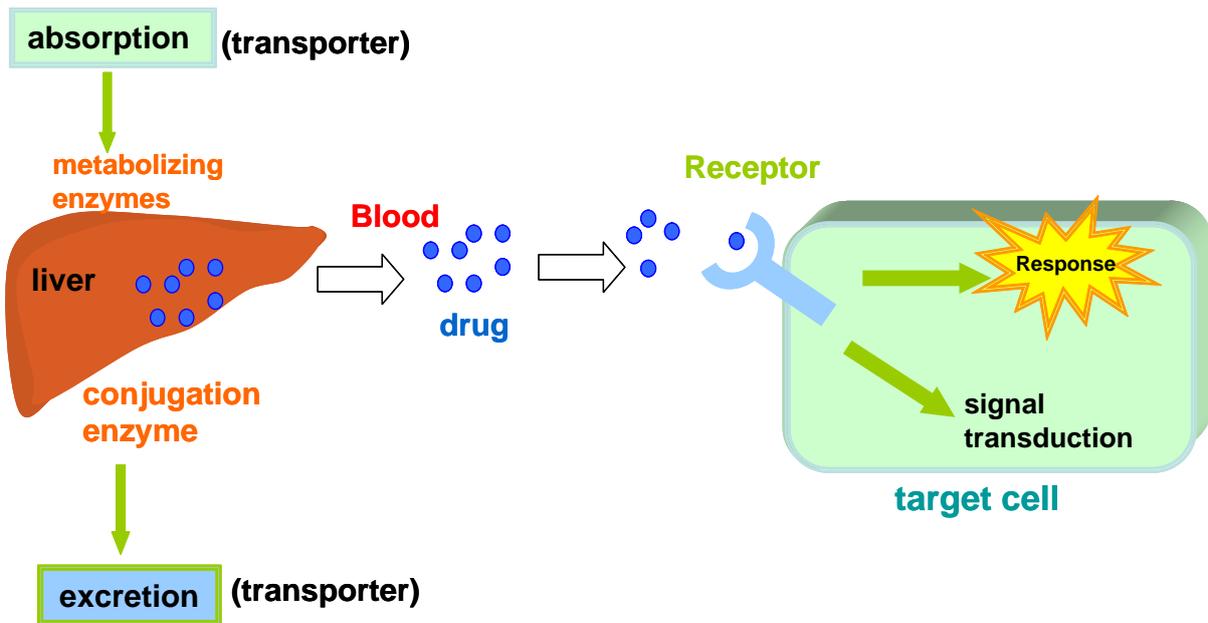


Figure 1. Inter-individual variation in drug response. Pharmacogenomics is the study of the role of inheritance in inter-individual variation in drug response. The field of pharmacogenomics began with a focus on drug metabolism, but it has been extended to cover the full spectrum of drug disposition, including transporters that influence drug absorption, distribution, and excretion. Furthermore, genetic variation in drug targets (*e.g.*, receptors) can have a profound effect on drug efficacy.

a drug, as opposed to pharmacodynamics, which is the study of what a drug does to a body. Pharmacogenomics influences both, so it can be used during all phases of clinical trials to assess safety and efficacy.

Pharmacogenomics is the study of the role of inheritance in inter-individual variation in drug response. The field of pharmacogenomics began with a focus on drug metabolism, but it has been extended to cover the full spectrum of drug disposition, including transporters that influence drug absorption, distribution, and excretion. Furthermore, genetic variation in drug targets (*e.g.* receptors) can have a profound effect on drug efficacy (Figure 1). However, the current state of clinical trials, and especially in the case of genotype-based subject stratification, is such that metabolizing enzyme-related studies are proceeding. Cytochrome P450 is a family of the body's more powerful detoxification enzymes. Of these, the CYP2D6, CYP2C19, and CYP2C9 genotypes are termed Known Valid Biomarkers, which are genetically associated with changes in drug effects and accepted by the scientific community as predicting clinical outcome.

Figure 2 shows a cumulative number of biomarkers investigated in the trials at this institute. CYP2C19-related studies are predominant in Japan. Research on CYP2C19 poor metabolizers in different ethnic populations has clearly revealed that the frequency of poor metabolizers is relatively high, approximately 20%, in the Japanese population. These genetic variations result in differences in kinetics and pharmacological action, *e.g.* clinical responses to proton pump inhibitors that are mainly metabolized by 2C19 in the liver (6).

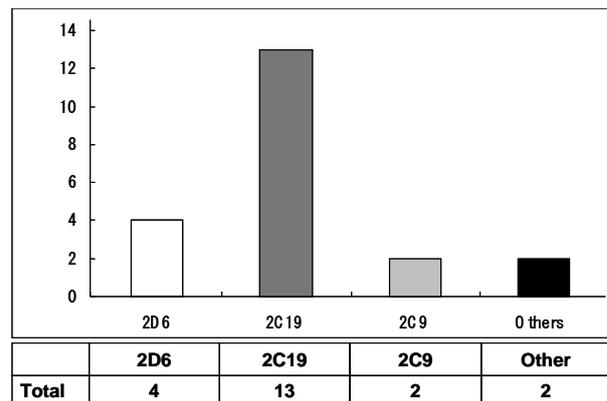


Figure 2. Biomarkers in PGx-related clinical studies. This bar graph shows a cumulative number of biomarkers investigated in trials at Sekino Clinical Pharmacology Clinic. CYP2C19-related studies are predominant in Japan.

3. Implementation of a novel, fully automated genotyping system

3.1. Research and development phase

A Research and Development laboratory has been established for in-house analysis of gene polymorphism. A novel automatic SNP-typing system has been developed in collaboration with ARKRAY, Inc, Kyoto, Japan. ARKRAY handles a wide range of analytical equipment for use in environments as diverse as major hospitals, diagnostic centers, and point-of-care testing. ARKRAY provides the latest equipment for major hospitals and diagnostic centers, easy-to-use, compact testing systems for clinics, and testing equipment for

convenient measurement at home or elsewhere for home care. The genotyping system that was developed is not a finished product but an experimental model. The most attractive features of this system are its compact design and automatic analysis including pre-treatment.

CYP2C19 is involved in the metabolism of several important groups of drugs including many proton pump inhibitors and antiepileptics. The CYP2C19 gene is located on chromosome 10q24. Twenty-one SNPs are found on CYP2C19. Within the Japanese population, however, variants except for *2 and *3 are absent. Thus, only these 2 non-functional alleles had to be genotyped. The *2 (m1) alleles (subtypes A and B) have a defining mutation of a G681 to A substitution that results in a splicing defect. Subtypes are not differentiated. The *3 (m2) allele has a defining mutation of a G636 to A substitution that results in a Trp212 to stop codon change.

In the current system, three-color real-time optical detection is possible. Thus, multiplex SNP genotyping can be performed at one time within 90 min. These results provided are consistent with the results obtained by allele-specific primer PCR as is conventionally used (7).

3.2. Overview of the newly finished product

Following improvements and upgrades, the system will be completed in the near future. It has a more compact design: 41 cm (width) by 45 cm (depth) by 41 cm (height) (Figure 3).

The system allows fully integrated automatic genotyping from sample pretreatment to gene amplification and signal detection. The equipment incorporates computer-free analysis so that measurement results can



Figure 3. Fully integrated and automatic genotyping system. Following improvements and upgrades, the system will be completed in the near future. It has a more compact design: 41 cm (width) by 45 cm (depth) by 41 cm (height).

also be analyzed with a single system. The working time has been reduced by using a newly developed technique for sample pretreatment that requires no DNA extraction. Following placement of the reagent pack and the sample, gene-typing results are available in 80 min.

3.3. Features and specifications

Key features

- Pretreatment, amplification, and detection are all automated.
- Automated testing procedures setup using bar-codes on the disposable reagent.
- Closed system for reagents.
- Rapid processing from preparation to detection. <Within 80 min>
- 4 independently programmable reaction sites.
- 3-color optical detection for each site.

Specifications

- EMC compliant
- Computer linkage *via* USB/Ethernet

3.4. The principle of signal detection

PCR is performed at the site with the SNP. The reagent contains Guanine Quenching Probes that have either of the complementary sequences of the target SNP. As the temperature is decreased following PCR, the probe and amplified product are hybridized regardless of whether a mismatch is present or not. Then, using a gradual temperature increase, the loosely bound mismatch sequences and probes detach and fluorescence is emitted. When the temperature is increased further, perfect match sequences and probes will detach and fluorescence strength will increase. In this way, an SNP can be detected by the difference in temperature and fluorescence. This method is known as 'Melting Point Analysis' or 'T_m Analysis' (Figure 4).

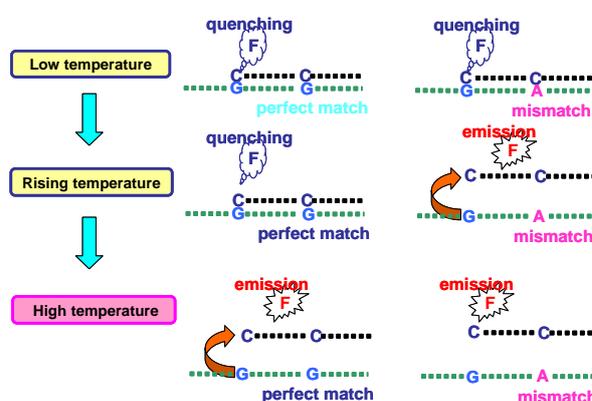


Figure 4. The principle of signal detection. SNPs can be detected by the difference in temperature and fluorescence. This method is known as "Melting Point Analysis" or "T_m Analysis".

Figure 5 shows an example of interpreted measurement results in the case of CYP2C19. With one pack of CYP2C19 reagent, the SNP in both *2 and *3 can be analyzed simultaneously. This example is of 'wild' in *2 investigation and 'hetero' or heterogeneous in *3 investigation, so the genotype was determined to be *1/*3.

3.5. Operation of the equipment

Prior to measurement, the necessary number of tips, reaction tubes and reagent packs are put in place. The equipment contains 4 independently programmable reaction sites (Figure 6). Reagent packs, reaction tubes, and tips are all included in one package. The

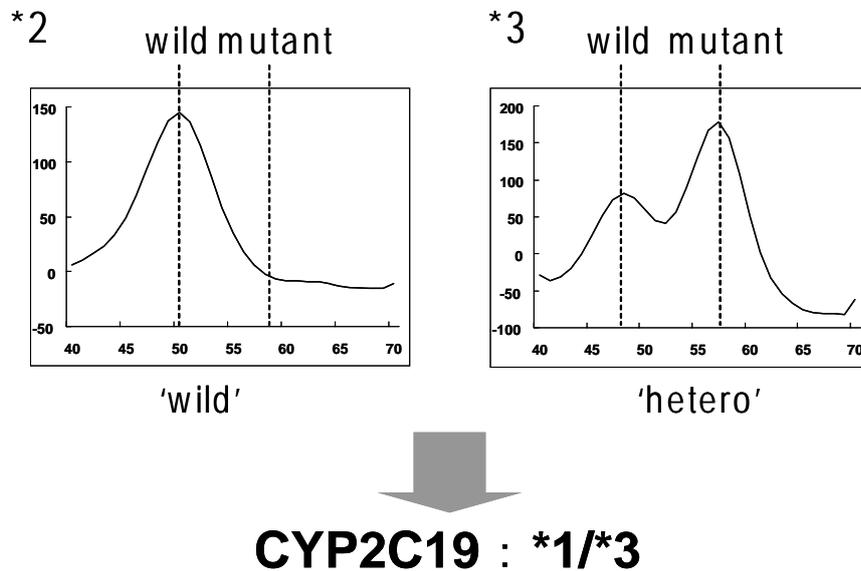


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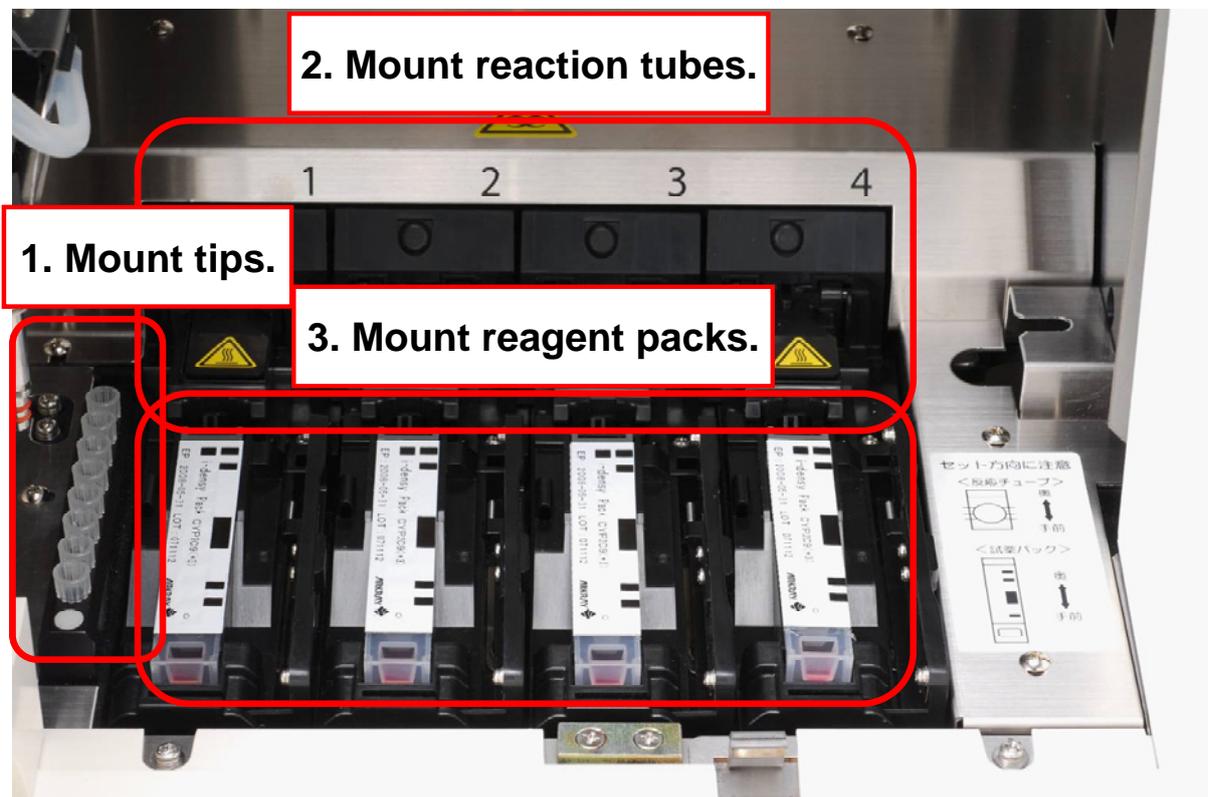


Figure 6. Operation of the equipment. Prior to measurement, the necessary number of tips, reaction tubes, and reagent packs are put in place. The equipment contains 4 independently programmable reaction sites.

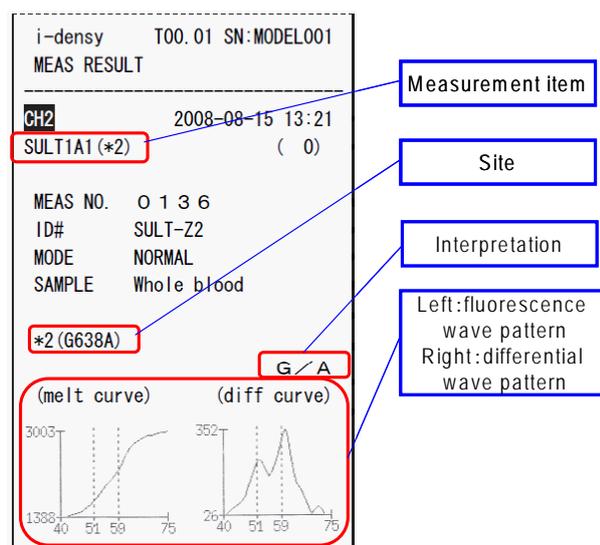


Figure 7. End of measurement. After measurement is complete, measurement results can be printed out. The equipment can store a maximum of 500 results per user. Previous results can also be printed.

reagent pack contains a solution for dilution and reagent for pretreatment, amplification and detection. Contamination can be avoided because each cell is designed to be separated so that the solution cannot flow into adjacent cells. The sample is applied to the sample block. In the current system, whole blood as well as oral swabs can be used as SNP-typing materials. When a blood sample is used, the sample block is filled with just 50 μ L of whole blood.

Measurement is started by simply pressing the start button. The system is equipped with automatic recognition using barcodes printed on the reagent packs and an easy-to-use color LCD with touch screen. These features allow rapid and simple gene-typing. After measurement is complete, measurement results can be printed out. The equipment can store a maximum of 500 results per user. Previous results can also be printed (Figure 7).

3.6. Forward-looking approach for clinical use

Figure 8 shows the prospective development of SNP-analysis. According to the order shown here, valid genomic biomarkers will expand from PK-related genes, *via* genes related to target receptors, to genes that cause disease in the future.

For example, disposition of warfarin provides an interesting model for a suitable clinical application of the system. Warfarin has received a great deal of attention in recent years as a target of personalized medicine (8). Warfarin is metabolized into inactive metabolites by CYP2C9, and warfarin inhibits the Vitamin K-dependent carboxylation of coagulation factor *via* vitamin K epoxide reductase complex 1 (VKORC1) (9,10). An extensive amount of clinical data suggests that the risk of bleeding is particularly

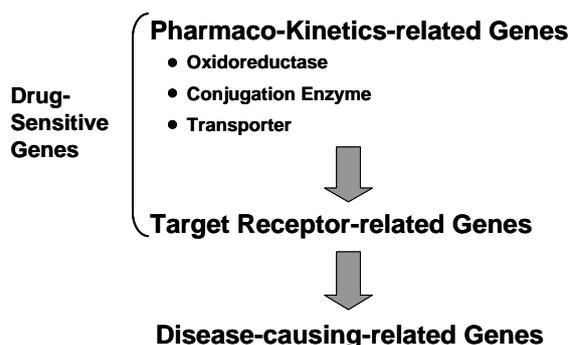


Figure 8. Prospective development of SNP-analysis. This diagram shows the prospective development of SNP-analysis. According to the order shown here, valid genomic biomarkers will expand from PK-related genes, *via* genes related to target receptors, to genes related to genes that cause disease in the future.

high in patients with gene variants in CYP2C9 and/or VKORC1 (11,12). In addition to the analytical program for CYP2C9, one has also been established for VKORC1. Preliminary investigations revealed data in general agreement with reference data. The system allows 2C9 and VKORC1-genotyping to be performed simultaneously, which can provide a clinically significant improvement to current practices. New software content related to gene polymorphism is currently being developing to meet the needs of clinical practice.

4. Conclusion

The SNP genotyping system thus developed is very unique in contrast to previous methods or systems for SNP genotyping. Rapid, simple and contamination-free genotyping system could contribute not just to PGx-related clinical trials but also to order-made therapy in the near future.

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