Chapter 2
Applications of Microarrays and Biochips in Pharmacogenomics

Gary Hardiman

Summary The complete sequence of the human genome and subsequent intensive searches for polymorphic variations are providing the prerequisite markers necessary to facilitate elucidation of the genetic variability in drug responses. Improvements in the sensitivity and precision of DNA microarrays permit a detailed and accurate scrutiny of the human genome. These advances have the potential to significantly improve health care management by improving disease diagnosis and targeting molecular therapy. Pharmacogenetic approaches, in limited use today, will become an integral part of therapeutic monitoring and health management, permitting patient stratification in advance of treatments, with the potential to eliminate adverse drug reactions. In this chapter, the current state of biochip technology is discussed, and recent applications in the arena of clinic diagnostics are explored.

Keywords AmpliChip; biochips; microarrays; P450; pharmacogenetics.

2.1 Introduction

The sequencing of the human genome has been widely touted as a critical scientific milestone that will revolutionize the process of drug discovery. The continuing analysis of the human genetic code will provide the scientific framework on which

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it may be possible to identify novel potential drug targets, the common genetic factors that can affect drug metabolism and toxicity, and the genetic factors that contribute to the wide variability in pharmacological treatment responses routinely observed in clinical settings. The ever-increasing utilization of genetic techniques, including microarray technologies, has provided a means by which geneticists, biologists, and pharmacologists have begun to bridge the gap between gene sequence and function. These newer approaches are currently under integration into multiple aspects of the drug discovery process. The use of genetic polymorphism analysis has been applied to target validation, pharmacokinetics and toxicology, and clinical pharmacogenomics, while microarray technologies have been utilized in target validation, *in vitro* pharmacology, and toxicology ***(1)***.

A DNA microarray (also referred to as gene or genome chip, DNA chip, or biochip) is a collection of microscopic DNA features attached to a solid support, commonly glass, plastic, or silicon. The array features or "spots" contain DNA probes that are used to interrogate individual genes or polymorphisms. Most arrays in use today contain hundreds to thousands of probes. The value of this technology is that it permits highly parallel measurements. In the case of gene expression profiling, the massive number of data points obtained from a single experiment provides insight into the state of a transcriptome in, for example, healthy and diseased cells or cells before and after exposure to a therapeutic treatment. The knowledge obtained from such comparisons is incredibly compelling as it permits the identification of gene families and pathways pertinent to the malady or drug treatment in addition to those that remain unaffected. Similar expression profiles may infer that genes are coregulated, enabling the formulation of hypotheses about genes with hitherto unknown functions by comparison of their expression patterns to well-characterized genes ***(2)***.

The applicability of microarrays in genomics research has expanded with the evolution and maturation of the technology. Biochips have found utility in exon-based gene expression analyses, genotyping and resequencing applications, comparative genomic hybridization studies, and genomewide (epigenetic) localization ***(3)***. Biochips are widely applied to improve the processes of disease diagnosis, pharmacogenomics, and toxicogenomics ***(4–7)***. In this chapter, the evolution of biochip platforms is reviewed; I compare and contrast platforms currently in use and discuss biochips in the context of pharmacogenetic testing.

2.2 Pharmacogenetic Testing and Health Care

Pharmacogenetics is the discipline that studies the relationship between a patient's inherited genetic makeup and that patient's response to pharmaceutical drugs. Pharmacogenetic testing aims at determining the underlying genotypic and phenotypic differences in the pharmacodynamics and pharmacokinetics of drug metabolism. Whereas pharmacogenetics refers to genetic differences (variation) in drug metabolism and response, *pharmacogenomics* refers to study of the multiplicity of

genes that ultimately determine drug behavior. Pharmacogenomics is in essence the whole-genome application of pharmacogenetics, correlating gene expression or single-nucleotide polymorphisms (SNPs) with drug efficacy and toxicity. Genetic variability in drug response occurs as a result of molecular alterations in the enzymes involved in the metabolism of a particular drug in addition to the drug receptors and transport proteins ***(8)***.

A recent advance and fundamental shift in health care has been the emergence of personalized medicine. Drug–drug interactions (DDIs) can have serious consequences, such as adverse drug reactions (ADRs), and extreme outcomes, including death. DDIs have become a serious issue, particularly in the care of elderly patients, who are often prescribed a wide variety of medications ***(9)***. ADRs are presently the fourth leading cause of death in the United States, resulting in 106,000 deaths per year, and the fifth leading cause of illness, resulting in 2.2 million hospitalizations annually. At present, approx. 28% of adults and 17% of children hospitalized have drug-related ADRs. The economics of drug-related morbidity and mortality has become a pressing issue, with current costs estimated at $177 billion annually ***(10)***.

Pharmacogenetic approaches, in limited use today, will in the near future become an integral part of the therapeutic monitoring and health management of patients. A major advantage of pharmacogenetic testing over classical therapeutic drug monitoring (TDM) approaches is that patient genotyping and stratification can be carried out in advance of drug treatments, thereby eliminating or reducing adverse effects. Testing can generally be performed in a noninvasive manner using DNA obtained from saliva, hair root, or buccal swab samples. Another benefit over traditional methods is that patient compliance with a particular treatment regimen is not required. In addition, the results remain constant over the lifetime of an individual, regardless of disease or aging. Finally, a major advantage of pharmacogenetic testing is that it can provide predictive value for many drugs rather than a single drug ***(8)***.

2.3 Important Pharmacogenetic Targets

The most relevant pharmacogenetic targets as defined by the American Association of Clinical Chemists (AACC) include the Cytochrome P450 enzymes CYP2D6, CYP2C9, CYP2C19, CYP3A5, CYP2B6 and thiopurine s-methyltransferase (TPMT), N-acetyltransferase 2 (NAT2), UDP glucuronosyltransferase 1 family, polypeptide A1 (UGT1A1), multi-drug-resistance (MDR1) gene and methylenetetrahydrofolate reductase (MTHFR). Drug metabolism occurs largely in the liver and involves cytochrome P450 (CYP450), a large family of oxidative enzymes. The name derives from “pigment at 450 nm” as the majority of family members possess red coloration owing to the presence of heme at the active site. Although CYP450 plays an important role in the synthesis and breakdown of hormones, cholesterol synthesis, and vitamin D metabolism, from a health care perspective its role in drug metabolism is its most pertinent. Most common variations in drug metabolism

between individuals can be explained by polymorphisms in the *cypP450* genes. One of the best characterized of the CYPP450 enzymes, CYP2D6, is responsible for metabolizing the majority of pharmaceuticals currently in use. These include an extensive range of therapeutic agents encompassing β-blockers, antidepressants, antipsychotics, and opioids. A poor metabolizer (PM) phenotype has been observed among 7–10% of the Caucasian population, with many suffering toxicity from normally prescribed doses. This is explained by adverse reaction to drugs prescribed in standard doses or undesirable DDIs when using multiple-drug therapeutics.

Warfarin (Coumadin) inhibits the synthesis of clotting factors, thus preventing blood clot formation. Although it remains the most frequently prescribed oral anticoagulant, it can cause severe bleeding that can be life-threatening and cause death. Successful management of warfarin therapy is problematical owing to the wide variation in drug response among patients. Variation in the vitamin K epoxide reductase complex 1 (VKORC1) gene affects the response to warfarin ***(11)***. Pharmacogenetic analysis of a patient's *CYP2C9* or *VKORC1* can provide information that allows fine-tuning of the appropriate warfarin dosage. Cytochrome P4502C19 metabolizes 15% of all prescribed drugs and is involved in the metabolism and clearance of antidepressants (tricyclic antidepressants [TCAs] and selective serotonin reuptake inhibitors [SSRIs]), anticonvulsants, anxiolytics, and benzodiazepines ***(12–14)***. For 2C19, two phenotypes with variable metabolic activity have been defined, the extensive metabolizer (EM) and poor metabolizer (PM). The PM phenotype is associated with low enzyme activity. East Asians are most likely to exhibit the PM phenotype, with 2C19 PM rates observed in up to 25%. CYP4503A4/3A5 is the most abundant CYP450 isoenzyme in humans and is responsible for the metabolism of the widest range of drugs. It is involved in the metabolism and clearance of calcium channel blockers, benzodiazepines, human immunodeficiency virus (HIV) protease inhibitors, HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase inhibitors, and antithrombolytics.

Thiopurine s-methyltransferase (TPMT) catalyzes the S-methylation or inactivation of the thiopurine drugs mercaptopurine, azathioprine, and thioguanine, which are commonly used to treat leukemia, rheumatic diseases, and inflammatory bowel disease. *TMPT* testing serves to detect patients at risk of developing side effects if treated with thiopurine drugs ***(12)***. *N*-Acetyltransferase 2 (NAT2) is of clinical importance as rapid or slow acetylation of therapeutic and carcinogenic agents is explained by variability at the *NAT2* locus. Interethnic variations in distribution of the acetylation phenotype are significant.

UDP glucuronosyltransferase 1 family, polypeptide A1 (UGT1A1), is a hepatic enzyme associated with the colorectal and small lung cancers. UGT1A1 metabolizes irinotecan, an antineoplastic agent utilized for the treatment of colorectal cancer. Pharmacogenetic testing for *UGT1A1* will help the optimization of therapeutic approaches with antineoplastic agents that inherently have a low therapeutic index and will spare patients from excessive toxicity resulting from therapy with irinotecan.

P-Glycoprotein (P-gp), a member of the adenosine triphosphate (ATP)-binding cassette family of membrane transporters, is encoded by the human multidrug-resistance (*MDR1*, *ABCB1*) gene ***(15)***. This integral membrane protein serves as an

energy-dependent drug efflux pump and reduces the intracellular concentrations of a wide range of drugs and xenobiotics. The overexpression of MDR1 is associated with resistance to doxorubicin, taxanes, and vinca alkaloids, which are used to treat cancer. Resistance to chemotherapy has become a major obstacle in anticancer treatment. Methylenetetrahydrofolate reductase (MTHFR) is a cytoplasmic enzyme that plays a role in the conversion of homocysteine (a potentially toxic amino acid) to methionine. A common 677TT genotype predisposes individuals to mild hyperhomocysteinemia (high blood homocysteine levels), which can lead to neural tube defects in offspring, arterial and venous thrombosis, and cardiovascular disease.

Currently, the methods employed for genetic testing are labor intensive and intricate and demand the concurrent analysis of multiple nucleic acid markers. Microarray technology is undeniably the most practical approach to multiplex and analyze biomolecular markers.

2.4 Evolution and Development of Microarrays

The origin of the microarray or biochip can be traced to a seminal publication by Edwin Southern over 30 years ago. Southern described a method by which DNA could be attached to a solid support following electrophoresis and interrogated for sequences of interest by hybridization with a complementary DNA sequence ***(16)***. The complementary DNA sequence, termed a *probe*, was labeled with either a radioactive or a fluorescent marker and hybridized to the DNA target sample, which was immobilized on a solid support, such as a nitrocellulose filter membrane.

The biochips widely in use today owe their existence to innovations in miniaturization, DNA synthesis and attachment chemistries, and improvements in image acquisition. Key pioneers in the early innovation and development of this technology were Hyseq (Sunnyvale, CA); Affymetrix (Affymax) (Santa Clara, CA); Oxford Gene Technologies (Oxford, UK); and Stanford University (Palo Alto, CA). Hyseq exploited oligonucleotide arrays to permit sequencing of target nucleic acid sequences. The complementary oligonucleotide probe sequences overlapped, permitting the discrimination of perfect match DNA hybrids from hybrids that contained a single-nucleotide mismatch ***(17)***. Affymetrix utilized very large scale immobilized polymer synthesis (VLSIPSTM) substrate technologies for the synthesis of both peptides and oligonucleotides on solid supports. They successfully applied this technology to DNA sequencing, DNA fingerprinting, chromosomal mapping, and specific interaction screening ***(18)***. Spotted microarrays, yet another widely utilized application of this technology, were pioneered at Stanford University by Patrick Brown and colleagues. These arrays are fabricated using a capillary dispenser, which deposits DNA at specific array positions. Spotted microarray production is highly automated, utilizing either capillary pin-based or ink-jet microdispensing liquid-handling systems ***(19,20)***.

The major commercial microarray platforms in use today, over ten years after their first description, include those from Affymetrix, Illumina, Agilent, and

Applied Biosystems. A detailed comparison and contrast of the salient features of each of these platforms has been described previously ***(21,22)***. The Affymetrix GeneChip™ has been the most extensively used owing to its extensive genome coverage, its ease of use, and its high level of reproducibility. It is comprised of short single-stranded oligonucleotides and is fabricated via a combination of photolithography and solid-phase DNA synthesis. Illumina (San Diego, Ca) has established a bead-based technology that was utilized initially for SNP genotyping and subsequently for gene expression profiling. These arrays are comprised of thousands of tiny etched wells, into which thousands to hundreds of thousands of 3-μm beads randomly self-assemble. Then, 50-mer gene-specific probes linked with "address or zip code" sequences are immobilized on the bead surface and are used to facilitate a decoding process, which maps a specific bead type containing a particular sequence to a given location on the array.

Applied Biosystems Expression Array System (Foster City, CA) has devised a chemiluminescence-based microarray platform utilizing 60-mer oligonucleotides which are validated offline by mass spectrometry and are subsequently printed onto a derivatized nylon substrate. Agilent Technologies (Palo Alto, CA) also utilizes 60-mers, which are synthesized in situ by ink-jet printing using phosphoramidite chemistry.

2.5 Microarrays and Genotyping

Single-nucleotide polymorphisms are highly abundant, with over 10 million present in the human genome, and they serve as valuable markers of genomewide variation. A chromosome region may contain many SNPs, but just a few "tag" SNPs are required to provide information on the pattern of genetic variation. The high costs associated with most SNP detection strategies have until recently made genomewide approaches impractical.

Illumina bead-based technology has been applied to both SNP genotyping and gene expression profiling applications and utilizes two distinct substrates, the Sentrix LD BeadChip and the Sentrix Array Matrix (which multiplex up to 8 and 96 samples, respectively). Genomewide genotyping of defined sets of hundreds of thousands of SNPs can be performed using one of two array types, the Infinium I 109 K SNP arrays or the Infinium II 317 K SNP arrays. A whole-genome amplification step is initially employed to enrich the target DNA up to 1000-fold. Once amplified, the DNA is subsequently fragmented and mobilized by hybridization to SNP-specific primers present on the array. In the case of the Infinium I assay, which utilizes an allele-specific primer extension approach, the DNA is hybridized to allele-specific primers that are extended with multiple labeled bases only if a perfect match exists between the target and SNP-specific probe ***(23)***. The Infinium II assay differs in that it is based on single-base extension (SBE). An oligonucleotide primer is hybridized adjacent to the SNP site and is extended with a single labeled dideoxy-nucleotide terminator corresponding to the minor or major allele. Genotyping calls can then be made based on the dye-labeled terminator that is incorporated ***(24)***.

2.6 Microarrays and Clinical Diagnostics

Microarrays are today applied in the clinical diagnostics and genotyping arenas. Their successful utilization and survival in the clinic will depend on the ability of the technology to meet the rigorous requirements applied to human diagnostics in a cost-effective manner.

2.6.1 Roche Diagnostics AmpliChip

The first pharmacogenetic microarray-based test approved for clinical use is the AmpliChip CYP450 from Roche Diagnostics (Basel), which measures genetic variation, both deletions and duplications, for the *CYP2D6* and *CYP2C19* genes. The AmpliChip is a marriage of expertise in polymerase chain reaction (PCR; Roche) and microarray (Affymetrix) technologies. The AmpliChip has been approved for *in vitro* diagnostic use in the United States and Europe. The test determines the associated predictive metabolizer phenotype (poor, intermediate, extensive, or ultra) and can aid physicians in individualizing patient treatment and dosing for drugs metabolized through these *P450* genes. It detects a total of 27 polymorphisms and mutations for the *2D6* gene and 3 polymorphisms for the *2C19* gene.

Once patient genomic DNA has been extracted, the test involves a series of five steps, and the analysis time from start to finish is 8 h. A minimum of 25 ng of input genomic DNA is required for the assay, and the preferred tissue source is blood, although buccal swab-derived DNA would also suffice. First, PCR amplification is carried out to amplify the genes of interest using gene-specific primers. This is followed by fragmentation and biotin labeling of the amplicons at their 3′ termini with terminal transferase (TdT). The biotin-labeled amplicon is subsequently hybridized to the AmpliChip DNA microarray. Following washing and staining via a strepavidin–phycoerythrin conjugate, the chip is scanned on an Affymetrix GeneChip Scanner 3000Dx, the data feature is extracted and analyzed, and genotyping calls are made.

2.6.2 Autogenomics BioFilm Microarrays

The Infiniti Analyzer, an automated, continuous-flow microarray platform for clinical applications has been developed by Autogenomics (Carlsbad, CA) ***(25)***. The underlying component of the Autogenomics technology is the BioFilm™, which consists of multiple layers of porous hydrogel matrices 8- to 10-μm thick on a polyester solid base. This provides an aqueous microenvironment that is highly compatible with biological materials. The BioFilm microarray is configured with 15 × 16 arrays (240 spots) per chip, suitable for current diagnostic applications, and permits analyses of both nucleic acid and proteins ***(26)***. It can be tailored to clinical genetic testing for custom polymorphisms of interest.

The analyzer integrates all the discrete processes of sample handling, reagent management, hybridization, and detection. A confocal microscope has been integrated into the analyzer; it has two lasers (red and green). In addition, a thermal stringency station and a thermal cycler for denaturing nucleic acids for primer extension studies or hybridization reactions in solution have been incorporated. A CYP2D6 assay has been designed to detect the most prevalent and informative *CYP2D6* allele variants ***(25)***. The target regions of the *CYP2D6* gene are amplified via a multiplex PCR reaction with specific primer and reaction conditions that can discriminate *CYP2D6* from its pseudogenes. The PCR multiplex reaction is followed by the incorporation of fluorescently labeled nucleotides via primer extension and hybridization of the labeled targets to immobilized oligonuleotides on the BioFilm. Other pharmacogenetic specific tests that can be carried out on this platform include, CYP2C9, CYP2C19, TPMT, CYP3A4/5, and NAT2.

2.6.3 Nanogen NanoChip™

An interesting development has been that of electronic chip technology. Nanogen (San Diego) developed the NanoChip™, which exploits the charged nature of biological molecules. Electronic charges can rapidly shift molecules from one location to another and concentrate them at defined sites on an array. The concentration of biological materials with electronics enables rapid hybridization reactions; instead of the 12 to 16 h traditionally required for passive hybridization, electronic hybridization reactions can be performed in 2 min. When a test site on the NanoChip is charged, a nucleic acid target rapidly moves to that site. Other sites, which are not charged, do not attract the target. Each site or feature can be individually charged electronically via platinum wires and can contain an individual assay or experiment. Electronic hybridization and stringency can be carried out with single-base resolution.

Nanogen has developed pharmacogenetics research reagents for the analysis of *CYP2C9* and *VKORC1*, mutations of which have relevance to warfarin dose optimization. The reagents can be used to rapidly determine genotypes for up to 78 patient samples. In November 2007, Nanogen announced it would be closing its microarray business and repositioning of the company with a focus on real-time PCR and point-of-care testing units.

2.7 Microarray Technology Limitations and Challenges

The commercial microarray platforms in use today have established efficiencies regarding signal dynamic range, the ability to discriminate related messenger RNA (mRNA) species, the reproducibility of the data (raw data, fold change and expression levels). However, technological and standardization limitations exist with

biochip technologies. Expression microarrays facilitate the analysis of the relative levels of mRNA species in one tissue sample compared to another. Although a measure of transcript abundance is achieved, biochips do not provide absolute quantification of the specific mRNA. Microarrays are further limited by the certainty that the data obtained merely indicate whether a given mRNA is above the system's threshold level of detection. If the signal is significantly above the background intensity, then one can say with confidence that the transcript is expressed in that tissue. However, the absence of signal does not indicate the lack of expression. It merely indicates that it is below the detection capability of the system, and there is still a probability that the mRNA is expressed, albeit at basal levels, and this low-level expression may be of biological relevance.

Expression analysis using DNA microarrays analyzes only the transcriptome; it should be mentioned that mRNA abundance in a cell often correlates poorly with the amount of protein synthesized *(27)*. Important regulation takes place at the levels of translation and enzymatic activities. The only effect of a signal transduction pathway that is observed in a gene expression experiment is that at the endpoint of a given pathway. DNA microarrays currently have little value in determining post-translational modifications, which influence the diversity, affinity, function, cellular abundance, and transport of proteins.

2.8 Conclusion

Currently, the methods employed for genetic testing are both labor intensive and highly complex and require the simultaneous analysis of multiple nucleic acid markers. Microarray technology is without doubt the most practical approach to multiplex and analyze biomolecular markers. Although widely used in the research setting, adaptation of microarray technology to the clinical environment has been slow.

The success of microarrays in the clinical laboratory will depend on their ability to adapt to the rigorous environment of routine usage while providing high-quality, reproducible, and robust results. The clinical environment stretches the limits of this technology as it measures performance criteria in a different manner compared to the research environment. One difference from an economic standpoint is that the cost per reportable result is more important than the cost per data point. Other key factors are the requirements for automation from sample processing to end result, precision, accuracy of results, and the ability to process large volumes of tests under strict regulatory guidelines and compliances.

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