Microfluidic Platform for Single Nucleotide Polymorphism Genotyping of the Thiopurine S-Methyltransferase Gene to Evaluate Risk for Adverse Drug Events

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Prospective clinical pharmacogenetic testing of the thiopurine S-methyltransferase gene remains to be realized despite the large body of evidence demonstrating clinical benefit for the patient and cost effectiveness for health care systems. We describe an entirely microchip-based method to genotype for common single nucleotide polymorphisms in the thiopurine S-methyltransferase gene that lead to serious adverse drug reactions for patients undergoing thiopurine therapy. Restriction fragment length polymorphism and allele-specific polymerase chain reaction have been adapted to a microfluidic chip-based polymerase chain reaction and capillary electrophoresis platform to genotype the common *2, *3A, and *3C functional alleles. In total, 80 patients being treated with thiopurines were genotyped, with 100% concordance between microchip and conventional methods. This is the first report of single nucleotide polymorphism detection using portable instrumentation and represents a significant step toward miniaturized for personalized treatment and automated point-of-care testing. (*J Mol Diagn* 2007, 9:521–529; DOI: 10.2353/jmoldx.2007.070014)

Single nucleotide polymorphisms (SNPs) account for 20 to 95% of interpatient variability in drug response,¹ offering the potential to identify prospectively individuals at risk of adverse effects and for individual tailoring of drug dosing. SNPs are the most common markers for both disease genes and drug response associations.^{2,3} Current prescription strategies are primarily empirical with

the same drug dose chosen for everyone, leading to adverse drug reactions and increased health care costs.⁴ Effective implementation of pharmacogenetics in the clinic requires testing of each patient at presentation to facilitate treatment decisions, and currently this is not routinely performed primarily because of limitations imposed by technology.

We developed microfluidic SNP genotyping platforms for the thiopurine S-methyltransferase (TPMT) gene.⁵ Small volumes used in microfluidic tests reduce reagent cost and time while allowing for higher sensitivity in detection by enhanced control of fluids, resulting in increasing levels of automation.⁶ TPMT is an important inactivator of thiopurine drugs used in the treatment of hematological and autoimmune diseases.⁷ Patients with inherited TPMT deficiency accumulate toxic metabolites in hematopoietic tissues, leading to severe hematological toxicity and potentially fatal neutropenia.⁸ The pharmacogenetics of TPMT were initially characterized in acute lymphoblastic leukemia.⁹ More recently, SNPs in TPMT have been shown to be significant confounding factors in the widespread use of thiopurines as immunosuppressants in the treatment of inflammatory bowel disease,¹⁰ rheumatoid arthritis,¹¹ atopic eczema,¹² and organ transplantation.¹³

TPMT activity is inherited as an autosomal co-dominant trait that exhibits genetic polymorphisms in all large populations studied.¹⁴ Approximately 90% of individuals have two functional TPMT alleles and high TPMT activity. Ten percent of individuals are heterozygotes, inheriting

Supported by the Canadian Institutes of Health Research (New Emerging Team Program, Institute of Genetics, to L.M.P. and C.J.B.), the Natural Science and Engineering Research Council (to L.M.P. and C.J.B.), the Western Economic Diversification (to L.M.P. and C.J.B.), iCore (to G.V.K.), the Department of Health, UK Pharmacogenetics program grant (TARGET study grant to S.P.), and the Alberta Heritage Foundation for Medical Research (to J.C.).

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Accepted for publication April 13, 2007.

L.M.P. is Canada Research Chair in Biomedical Nanotechnology. J.C. and G.V.K. hold awards from the National Research Council's National Institute of Nanotechnology and a Canadian Institutes of Health Research Translational Research Training Program in the Department of Oncology.

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one nonfunctional mutant allele and intermediate TPMT activity. Of the population, 0.3% have low or no detectable enzyme activity because they inherit two nonfunctional TPMT alleles.¹⁵ TPMT-deficient patients at risk for hematological toxicity can tolerate a 10- to 15-fold reduction in thiopurine dose.¹⁶ Three predominant alleles (TPMT*2, TPMT*3A, and TPMT*3C) account for more than 95% of low-enzyme activity cases.¹⁴ The 238G>C, 460G>A, and 719A>G SNPs, which account for the three signature alleles, are nonsynonymous SNPs that alter the TPMT amino acid sequence, resulting in enhanced TPMT proteolysis and a reduction in enzyme activity.¹⁷

Despite the widespread use of thiopurine drugs, prospective TPMT genotyping has yet to be established as routine clinical practice.^{18–20} Although there are numerous methods to genotype SNPs,²¹ most require substantive infrastructure, highly trained operators, and batch processing of many samples. Several microchip-based techniques have been demonstrated for mutation detection, but most involve large-scale genotyping or mutational screening^{22–28} and some off-chip processing. Work to date^{1,3–5} has primarily used expensive glass microchips in which mainly capillary electrophoresis (CE)-based functionality is included. Here, we demonstrate genotyping using disposable poly(dimethylsiloxane) (PDMS)-based polymeric devices. There are limited demonstrations of mutation detection using polymeric devices.^{29–31} Ng and Liu³² reviewed multiple approaches, including microarray,³³ bead-based microfluidics,³⁴ and microelectrophoretic platforms³⁵ for rapid SNP detection in microliter volumes.³² However, currently available platforms require extremely large sample sets (eg 10,000)³⁶ for cost effective testing. Such large batch processing is not feasible in a clinical setting, particularly for rare diseases such as acute lymphoblastic leukemia. Batch processing imposes unacceptable delays in the initiation of therapy. High-throughput platforms require complex fabrication,³² extensive and expensive infrastructure,³⁶ and highly trained staff. TPMT genotyping using conventional approaches has been demonstrated by a number of techniques.^{37–40} Polymerase chain reaction (PCR)-based methods initially designed to genotype a small number of samples³⁶ are more suitable for clinical diagnostics than high-throughput detection methods developed for discovery, screening, or validation of candidate SNPs.

Restriction fragment length polymorphism (RFLP) and allele-specific polymerase chain reaction (AS-PCR) are inexpensive genotyping methods using specific primers and restriction enzymes to detect specific DNA sequences. RFLP, which has been implemented on existing microfluidic platforms,^{24,31} uses restriction enzymes to digest DNA at specific sequences in an amplicon flanking the SNP of interest. RFLP is widely used to perform SNP genotyping, and in computational models is applicable to up to 85% of SNPs in the National Center for Biotechnology Information database, (dbSNP).⁴¹ AS-PCR exploits the high fidelity of DNA polymerase to elongate only in the presence of an exact match of two allele-specific primers that anneal to a SNP at its 3' end.

The well-characterized pharmacogenetics of TPMT and the widespread use of clinical thiopurines make SNP genotyping of TPMT a highly relevant model to demonstrate the proof of principle for adapting PCR-based SNP detection strategies to microfluidic platforms for clinical diagnostic testing. This chip-based miniaturization provides a clinically feasible genotyping platform for one-at-a-time testing.

Materials and Methods

Patient Samples

Patient DNA samples used in this study were from the pretrial validation phase of the TPMT: Azathiopurine Response to Genotyping and Enzyme Testing (TARGET) study, an in-progress randomized controlled trial to assess the clinical utility and relative cost effectiveness of the TPMT pharmacogenetic test for patients treated with azathiopurine for inflammatory conditions in the UK. Blood samples (5 ml) were collected from 62 patients with inflammatory bowel disease after informed consent and ethical approval. All patients were recruited from the Gastroenterology Department at the Manchester Royal Infirmary, Manchester, UK. DNA extraction was performed at National Genetics Reference Laboratory (Manchester, UK), using Autopure LS large sample nucleic acid purification instrument (Gentra Systems, Minneapolis, MN) according to the manufacturer's protocol and was stored in Nunc vials (Nalge Nunc, Hereford, UK) at –20°C. DNA was quantitated using RNase P assay on a 7900 HT fast real-time PCR system (Applied Biosystems, Warrington, UK) according to the manufacturer's protocol and was normalized to a concentration of 30 to 50 ng/μl. Samples were blinded and tested in an anonymous manner. Positive controls for homozygous TPMT SNPs were supplied by the Evans (St. Jude's Hospital, Memphis, TN), Ashen (University Hospital Goettingen, Goettingen, Germany), and Zwicker (Institut für Klinische Pharmakologie, Bremen, Germany) laboratories and confirmed by direct sequencing before use.

Conventional RFLP

The RFLP protocol for TPMT¹⁵ has been frequently used.⁴² However, the published protocols^{15,43} use significant amounts of DNA and purification steps before digestion. To adapt these protocols on-chip, the published protocols were streamlined as described below, validated in a conventional approach, and then further adapted to the microchip.

All primers and restriction enzymes used for RFLP are listed in Table 1. For the 238G>C SNP, primers designed to flank the SNP were used to create a 293-bp PCR product,⁴³ but novel PCR conditions used an 8× reduction in DNA, without purification of PCR products before restriction digestion. Thermal cycling conditions were as follows: initial denaturing at 95°C (5 minutes); 30 cycles of DNA denaturing, 95°C (30 seconds); primer annealing, 60°C (30 seconds); dNTP polymerizing, 72°C (30 sec-

Table 1. Primers and Restriction Enzymes for RFLP

SNP	Forward primer	Reverse primer	Restriction enzyme
238G>C	5'-CTTTGAAACCCCTATGAACCTGATTT-3'	5'-CCCAAATCAAAACAAACCTTAAAT-3'	BslI
460G>A	5'-ATAACAGAGTGGGGAGGCTGC-3'	5'-CTAGAACCCACAAAAGTATAG-3'	MwoI
719A>G	5'-CAGGCTTTAGCATAATTTCAATTCCT-3'	5'-TGTTGGGATTACAGGTGTGAGCCAC-3'	AccI

onds); and final extension at 72°C (10 minutes). The PCR reaction mixture contained a final concentration of 1× PCR buffer, 2.0 mol/L MgCl₂, 200 nmol/L of each dNTP, 0.2 μmol/L of each primer, and 10 ng of genomic DNA. Amplification used 0.5 U (0.1 μl) of Platinum TaqDNA polymerase (Invitrogen Life Technology, Carlsbad, CA). After PCR, the PCR product was incubated at 55°C for 1 hour using 2.5 U (0.25 μl) of BslI (New England Biolabs, Ipswich, MA) and a volume of 10× NEBuffer 3 (New England Biolabs) to bring the final concentration of the mixture to ×1. BslI digestion of mutant DNA yields 142-bp and 138-bp fragments, whereas wild-type DNA lacks the restriction site and remains undigested (see Results). Digested products were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining.

For the 460G>A SNP, a similar RFLP assay was performed using previously reported primers¹⁵ except the final concentration of MgCl₂ in the PCR mixture was 2.5 μmol/L and the restriction digest was performed with MwoI and NEBuffer 3 for 60 minutes at 60°C. MwoI digestion of wild-type DNA yields 267-bp and 98-bp fragments whereas mutant DNA lacks the restriction site and remains undigested at 365 bp.

For the 719A>G SNP, the same RFLP assay was performed using previously reported primers,¹⁵ except the final concentration of the PCR mixture contained 4.0 μmol/L of MgCl₂ and 0.02 μmol/L of the forward primer. The restriction digest was performed with AccI and NEBuffer 4 for 2 hours at 37°C. AccI digestion of mutant DNA yields 207-bp and 86-bp fragments, whereas mutant DNA lacks the restriction site and remains undigested at 293 bp.

Microchip RFLP

On-chip RFLP was performed on three-port microfluidic chips made of patterned PDM irreversibly bonded to glass.⁴⁵ A PCR reaction (~2 μl) was performed in the same conditions discussed above except that the amount of Taq polymerase was increased 2.5 times to 1.25 U (0.25 μl), and the forward primers used were labeled with a 5' fluorescent dye, either a VIC dye (Applied Biosystems, Foster City, CA) or Cy-5 dye (Integrated DNA Technologies, Coralville, IA), depending on the analysis system as described below. After PCR, restriction digest was performed on the same microfluidic chip with the addition of 2.5 U of the specified enzyme. The BslI and MwoI on-chip digestions were performed at room temperature for 10 minutes, whereas the AccI digest required incubation on the chip at 37°C for 2 hours. After digestion, restriction fragments were identified by microchannel CE on a separate microchip.

Conventional AS-PCR

As with RFLP, published protocols¹⁵ for AS-PCR were further optimized in novel conditions to port the protocol to the microchip. Allele-specific primers for the 238G>C were used as previously reported,¹⁵ using only 50 ng of genomic DNA for each reaction, instead of the 400 ng required for conventional analysis.¹⁵ Each sample was separately amplified with the forward wild type, 5'-GTATGATTTTATGCAGGTTTG-3', or mutant primer 5'-GTATGATTTTATGCAGGTTTC-3' with a common reverse primer, 5'-TAAATAGGAACCATCGGACAC-3'. The amplification occurred with the wild-type primer only in the presence of wild-type DNA (G238). Amplification occurred for the mutant primer only in the presence of mutant DNA (C238) producing a 256-bp product. Product identity was confirmed by sequencing.

Integrated Microchip PCR and CE for AS-PCR

AS-PCR was performed on a patterned PDMS microchip.⁴⁴ The single chip (PCR-CE chip) integrates microchip PCR with CE using pinch-off valves to manipulate fluids as previously reported.⁴⁵ On-chip AS-PCR in a volume of ~2 μl was performed in similar conditions as described with the same modifications for on-chip RFLP. After amplification, a 1-μl aliquot of the PCR product was pumped from the enclosed PCR chamber into the open CE injection well containing the above-mentioned mixture, and 1 μl of DNA size standard was added, followed by electrophoretic separation, as described elsewhere.⁴⁵

TaqMan SNP Genotyping

TPMT SNP genotyping was performed by TaqMan 5' allelic discrimination assay (Assays-by-Design; Applied Biosystems) according to the manufacturer's instructions. Each PCR reaction contained 2.5 μl of TaqMan Master Mix, 0.25 μl of 40× assay mixture, and 2.25 μl of distilled water. Five μl of this reaction mix was added to 30 ng of DNA in a 96-well plate (ABgene Ltd., Epsom, UK), and PCR was performed using the following reaction conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. On completion of the reaction, genotype analysis was performed using the Sequence Detection Software version 2.2.2 (Applied Biosystems), using the allele discrimination option and interpretation of genotypes were made.

Figure 1. Schematic of the microfluidic chip (a), and methodology of SNP testing within the microfluidic platform. Stage 1 consists of fluid handling for thermal cycling (b) and subsequent enzymatic digestion within the PDMS/glass disposable microchip. In stage 2, detection of the fluorescently tagged PCR product is performed for the RFLP and AS-PCR methodologies within commercial electrophoresis equipment (c). Comparable results in detection were also achieved from the custom-built inexpensive electrophoresis equipment (d). Eventually, this equipment will have all fluid handling and thermocycling functionalities, thus leading to a fully independent and portable platform.

Microchip Heating Instrumentation

A custom-built dual thermoelectric module (Peltier-based) temperature cycling system provided for rapid temperature transitions during both heating (5 to 6°C/second) and cooling (3 to 4°C/second) along with stable hold temperatures ($\pm 0.1^\circ\text{C}$ of the set point). The thermoelectric modules are physically arranged in a cascade mode for improved performance and stacked between highly pure copper plates to ensure uniform spreading of the heat across the thermoelectric module (Figure 1b). The current flow through the thermoelectric modules regulates the heat-flow and thus the temperature in the chip. To ensure the eventual performance of thermal cycling with stable hold times and rapid transitions, this current flow is regulated by a custom-built proportional integral derivative controller, which is software based and resides in the microcontroller within the drive electronics. Further details are reported elsewhere (G. Kaigala, J. Jiang, C.J. Backhouse, H. Marquez, submitted). Fluid handling is performed using robotic arms that make use of the elastic nature of PDMS; thus by externally applying pressure at the valving points, the fluid is confined during thermal cycling within the PCR chamber. Further details about fluid handling and microchip fabrication can be found in Kaigala and colleagues.⁴⁵

Microchip CE

Fragment analysis of 5' VIC-labeled DNA was performed on glass microchips, unless otherwise specified, using

the Microfluidic Toolkit μTK (Micalyne, Edmonton, AB, Canada) by laser-induced fluorescence with excitation of 532 nm and detection at 578 nm.⁴⁶ Channels were filled with POP-6 polymer (Applied Biosystems, Foster City, CA) heated to 65°C for 10 minutes. The CE sample loading well is filled with a mixture of 1 μl of the digested PCR product, 0.5 μl of a fluorescently labeled DNA ladder, GeneScan 500 TAMRA (Applied Biosystems), 1.2 μl of HiDi formamide (Applied Biosystems), and 0.3 μl of a 1 $\times$ genetic analysis buffer with ethylenediamine tetraacetic acid (GABE; Applied Biosystems), which was denatured at 96°C for 5 minutes and rapidly cooled to $\sim 4^\circ\text{C}$. Sample injection with 0.4 kV (60 seconds) followed by separation at 6 kV (240 seconds) was performed.⁴⁶

Portable CE System

The portable CE system performs electrophoresis of Cy5-labeled DNA with the same parameters as a commercially available microchip-based CE system, the μTK . The maximum potential for electrophoresis is 6 kV for the separation of the DNA fragments and size standards. The wavelength for excitation is via a laser diode at 635 nm, with emission detection at 670 nm. The optics have been simplified compared with the μTK , using a single emission filter and by shining the laser diode directly onto the channel rather than through a dichroic. Fluorescence detection is done using a digital camera (charge-coupled device). Image processing is performed externally on a computer to produce an electropherogram—the fluorescence versus time is extracted from a series of digital photographs, requiring a sampling period of less than 1 second when electrophoresis is performed for 6-kV experiments. In this system's current state of development to increase signal intensity, PCR product was concentrated off-chip. Further improvements in the sensitivity of the portable CE system are underway.

Results

Microchip RFLP

The microchip architecture and instrumentation used for genotyping are shown in the diagram of Figure 1. The TPMT genotype was determined in a population of 80 patients undergoing thiopurine immunosuppression. A set of known variants was added to this population. All genotypes were blinded until results were obtained, followed by comparison between microchip RFLP (Figure 2A) and conventional RFLP. For this study, as the external gold standard, a commercially available TaqMan assay was performed according to the manufacturer's protocol at the National Genetics Reference Laboratory, Manchester, UK. Conventional RFLP, as a control for the intermediate step, produced restriction fragment patterns on gel electrophoresis as expected from previous reports (Figure 2B). Microchip RFLP produced electropherograms with patterns of fluorescent intensity peaks (Figure 2C) similar to band patterns on gel electrophoresis, enabling clear genotyping based on fragment size. Restriction

Figure 2. A1–A3: Schematic diagram of RFLP genotyping. Primers flanking the SNP of interest amplify a PCR product that was then interrogated by the restriction enzyme producing specific fragments. B1–B3: Typical band pattern on agarose gel electrophoresis of conventional RFLP. C1–C3: Representative electropherograms from microchip CE with fluorescence measured by arbitrary fluorescence units (y axis) against time (x axis).

tion digest fragments in all cases were of greater or equal fluorescence intensity to DNA size standards facilitating rapid and easy determination of restriction fragment patterns even with the monochromatic detection system of the μ TK.

Among the 80 patients tested by microchip RFLP, one was heterozygous for the 238G>C SNP (TPMT*2), and five were heterozygous for both the 460G>A SNP and the 719A>G SNP (TPMT*3A). No homozygotes were detected for any of the three signature SNPs among the population set studied. Microchip RFLP detected homozygotes for all three SNPs in DNA from known positive controls, derived from patients known to be deficient in TPMT activity. Table 2 shows a comparison between

microchip RFLP, conventional RFLP, and TaqMan demonstrating 100% concordance between all of the methods. In comparison with gold standards, microchip RFLP matches the sensitivity and specificity for genotyping TPMT SNPs in clinical samples.

Microchip-Based Integrated AS-PCR-CE

To demonstrate SNP genotyping with a single biochemical reaction on an integrated microchip, AS-PCR was used to successfully genotype (in duplicate) the 238G>C SNP. DNA polymerase only elongates in the presence of an exact match between the primer and the template, particularly at the 3' end. AS-PCR uses this high fidelity of DNA polymerase with two allele-specific primers, the 3' nucleotide of which anneals to either the wild type or mutant allele. These allele-specific primers were used in two separate PCR reactions with a common reverse primer. Amplification in the presence of the specific primer indicates the genotype. The specificity of allele-specific primers was tested on-chip with known positive controls to confirm the amplification of PCR product by a given primer only in the presence of the corresponding template (Figure 3). This test was performed on an integrated microchip with both PCR and CE functionality (Figure 1). Results from the integrated PCR-CE chips produced electropherograms (Figure 3C) clearly showing the expected product peak at 256 bp, as sized relative to the DNA ladder. For homozygotes (w/w and mt/mt), there is a significantly more intense product peak compared with the heterozygote (wt/mt). The weaker intensity peaks in the heterozygote are as expected because there is approximately half as much template for each allele; however, the product peak remains clearly distinguishable from size standards, enabling reliable allele calls.

Portable Microchip CE

Using a newly developed portable CE system (Figure 1), we analyzed purified RFLP products to demonstrate the potential of this system for clinically based genotyping (Figure 4). For all three variants, we find sufficient resolution in separation, and appropriate sizing with monochromatic detection. This has been demonstrated in the discrimination capability between alleles (mt/mt, wt/mt, w/w) of the 238G>C SNP (Figure 4). Using AS-PCR,

Table 2. Comparison of Genotyping Results between Conventional RFLP, Microchip RFLP, and TaqMan Performed at St. Mary's Hospital for Patients Enrolled in the TARGET Study

	238G>C			460G>A			719A>G		
	Wt/Wt	Wt/Mt	Mt/Mt	Wt/Wt	Wt/Mt	Mt/Mt	Wt/Wt	Wt/Mt	Mt/Mt
Conventional RFLP	79	1	0	73	7	0	73	7	0
Microchip RFLP	79	1	0	73	7	0	73	7	0
TaqMan	79	1	0	73	7	0	73	7	0
Concordance	100%	100%	100%	100%	100%	100%	100%	100%	100%

More than 200 microchip runs were performed to analyze the samples used here for genotyping. Replicate runs were always consistent with each other. All runs included positive controls for each genotype and negative controls lacking the DNA template to monitor for contamination. All samples were run and the electropherograms were analyzed for allele calls in a blinded manner.

A. Schematic of AS-PCR

B. Conventional AS-PCR on gel

C. Microchip AS-PCR

Figure 3. **A:** Schematic diagram of AS-PCR. Two separate PCR reactions with allele-specific primers were performed for each sample. Amplification only occurred when sample template matches primer. **B:** Typical result from agarose gel electrophoresis of conventional AS-PCR. **C:** Representative electropherograms from microchip CE with fluorescence measured by arbitrary fluorescence units (y axis) against time (x axis). Size standard and product peaks are as labeled.

homozygotes and heterozygotes are clearly distinguishable from size standards.

Discussion

Microchip RFLP

As compared with conventional published protocols, microchip RFLP uses eightfold reduced levels of patient DNA and requires no purification steps between PCR and restriction digest. The original studies by Yates and colleagues¹⁵ (and other subsequent studies) describing RFLP genotyping of TPMT used up to 400 ng of DNA whereas the microchip methods described in this study require at most 50 ng of DNA. Reduction in template to 50 ng of DNA is representative of most other PCR-based applications. Compared with conventional RFLP, which typically requires an additional purification step after PCR and 1-hour incubation at elevated temperature, *Bst*I and *Mwo*I digestion were performed directly on the same

Figure 4. Electropherograms of RFLP genotyping of the 238G>C SNP for the TPMT *2 mutant allele performed on a portable microchip CE system. Fluorescence extracted from integrated charge-coupled device camera intensity values (y axis) against time (x axis). Size standard and product peaks are as labeled. PCR product was concentrated off-chip before use of the portable system. mt/mt: a single fully digested product peak at 142 bp indicates a homozygote mutant. mt/wt: a full-length product peak at 281 bp and a digested product peak at 142 bp indicate the presence of both alleles. A heterozygote. wt/wt: A single full length product peak at 281 bp indicates a homozygote wild type.

chip without purification, at room temperature, in 10 minutes. This saving in time suggests that 10 minutes for digestion is optimal to sufficiently digest DNA and to detect restriction fragments, thereby reducing the overall assay time. Further acceleration of the overall assay protocol on microchip occurs during on-chip CE, which takes seconds rather than the minutes to hours required for conventional CE or slab gel electrophoresis. With the current chip-based analysis, PCR-RFLP/CE takes 3 hours, as compared with at least 9 hours using conventional testing. With further developments, we envisage

completion of entire microchip-based testing in minutes. Although, microchip PCR has been demonstrated at considerably smaller volumes,^{47,48} SNPs are present on 50 to 100% of all DNA templates in a given individual, with abundant DNA available from peripheral blood, making small volume PCR less compelling. Furthermore, the size of restriction digest fragments resolved in the present work, 87 bp to 365 bp, demonstrates that a wide range of fragment sizes can be resolved on-chip. The close proximity of the fluorescently labeled primer for the 719A>G SNP demonstrates the versatility to resolve restriction digest fragments smaller than 100 bp by microchip CE. This is a first step toward establishing the use of mismatch primers⁴⁹ with an internal mismatch to the template that create an artificial restriction site for SNPs having no natural restriction site.

Microchip AS-PCR

Beyond the PCR, AS-PCR requires no biochemical reactions to interrogate a SNP thus reducing the need for downstream reagents and incubation times. Furthermore, integration of PCR and CE eliminates the time required for an operator to manually transfer PCR products from one platform to another. Ideally, the speed of AS-PCR is limited only by the speed of thermocycling and CE. In the present work, we demonstrate a rapid CE (requiring seconds). With future advances in microchip PCR, genotyping by AS-PCR may be realizable within minutes. In a clinical setting, a rapid pharmacogenetic test would allow for the immediate initiation of therapy without the need for delays inherent in batch processing methods that require a subsequent follow-up visit. This testing strategy provides a yes/no answer. Because SNPs are present in all cells of the body, there is no need for quantitative assays. In the future, multiplex AS-PCR has the potential for simultaneous testing at a reduced cost of multiple SNPs through the use of creative primer design and tagging.

Portable Microchip CE

The resolution in separation of the portable CE system compared favorably with a commercially available microchip-based instrument (μ TK) and is more than sufficient for a confident allele call using RFLP. Multicolor detection systems are being developed to detect PCR products from multiplexed reactions along with features integrated for performing thermal cycling within this system to eliminate the manual transfer of the chip from the thermal cycling platform to the CE platform, for truly point-of-care diagnostics.

Concluding Remarks

Microfluidic chip-based genotyping of SNPs offers the potential for a rapid and cost-effective method for prospective clinical genotyping in the clinic and for personalized drug therapy. The present work demonstrates the

adaptation of conventional methods to miniaturize microfluidic platforms, thus ultimately eliminating the need for a highly trained operator. Compared with conventional RFLP, microchip RFLP performs a genotype of the signature TPMT SNPs, with less initial DNA, with no need for a purification step between PCR and enzyme digestion at room temperature, and with subsequent identification of products in seconds by microchip CE. AS-PCR on an integrated PCR-CE chip demonstrates single-step genotyping on a single platform eliminating the need for downstream reactions to discriminate alleles. As instrumentation is improved in future, the central biochemistry of RFLP or AS-PCR demonstrated in the present work offers the potential for genotyping within minutes.

TPMT gene analysis demonstrates that although the relevant genes and polymorphisms underlying differential drug metabolism are known, genotyping would be clinically valuable, and the genotyping of those polymorphisms would be cost-effective for the health care system, and actual patient testing is impaired by the lack of clinically feasible technologies. The development of high-throughput SNP testing systems does not address the need for rapid, inexpensive, and accurate clinical testing of a single, stand-alone patient. Rather, their complex designs, although increasing functionality, add to the cost of fabrication, operating, and maintenance of these systems.

Microchip-based methods offer more rapid analysis than conventional methods of RFLP and AS-PCR. They are less expensive and more portable than TaqMan protocols. Although reagent costs for TaqMan genotyping of one SNP are ~20 cents per sample, this is possible only with extremely large sample sets (10,000 to 25,000)³⁶ that for reasons discussed above are not suitable for clinical testing and does not include labor or platform costs. Although it is difficult to approximate the cost for conventional testing of a single or limited number of patients on such a system, it is likely to be cost prohibitory. At present, the cost of chips and reagents for a stand-alone genotype of a single patient for the three TPMT signature SNPs using microchip-based methods is less than \$10, with instrumentation currently costing less than \$1000. These costs are anticipated to decrease dramatically in the future as microchip platforms are optimized and fabricated on a larger scale. In contrast, the costs of conventional systems are ~50 to 100 times higher.

At present, the rate-limiting step for all genotyping methods, conventional or microchip based, remains the sample preparation of whole blood. We have developed methods to perform sample processing and DNA purification on-chip (in preparation), and work is in progress to integrate sample-processing modules with PCR/CE modules on the same chip.

In summary, pharmacogenetic SNPs have been identified by an entirely chip-based genotyping system. On-chip testing has been validated using patient samples, in comparison with conventional technologies. This work demonstrates that simple biochemistries first developed to test small numbers of patients can be adapted to

miniaturized platforms and portable instrumentation for their use in a clinical setting.

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