MEETING REPORT

Approaches for Analyzing Human Mutations and Nucleotide Sequence Variation: A Report from the Seventh International Mutation Detection Meeting, 2003

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The Seventh International Symposium on Mutations in the Human Genome, Mutation Detection 2003, was held during 2–6 July 2003 in Palm Cove near Cairns, Australia. The meeting was organized under the auspices of the Human Genome Organisation (HUGO) as a satellite meeting of the International World Congress of Genetics, held in Melbourne the following week. Meeting participants reported on advances in mutation detection technologies, including advances in high-throughput detection systems for SNP genotyping applicable to the international haplotype mapping project (HapMap); and bioinformatics tools, including databases for handling and processing growing amounts of genome variation data. This meeting report summarizes the presentations and cites related articles from the special issue of Human Mutation (Volume 23\#5, May 2004; available online at www.wiley.com/humanmutation). Hum Mutat 23:401–405, 2004. © 2004 Wiley-Liss, Inc.

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INTRODUCTION

Every other year, scientists from all over the world who develop or use methods for analyzing human mutations get together to discuss progress of their field. The first of the International Mutation Detection meetings held in Oxford, UK, in 1991, was followed by biannual meetings at beautiful locations throughout Europe. The seventh Mutation Detection meeting was held from 2–6 July 2003; this time, however, it was located outside of Europe, in Palm Cove near Cairns, Australia, a beautiful site where the tropical rain forest of Northern Australia meets the Great Barrier Reef. The meeting was organized under the auspices of the Human Genome Organisation (HUGO, London, UK) and was a satellite meeting of the International World Congress of Genetics held in Melbourne the following week. The timely topic of how to analyze human genomic sequence variation and its consequences, combined with the fantastic site of the meeting, attracted 170 scientists from 24 countries. Meeting participants included both mutation detection “old-timers,” as well as a large number of “newcomers” to the field. Traditionally, the atmosphere of the Mutation Detection meetings has been relaxed, with ample time for informal discussions, and this pleasant atmosphere prevailed at the Palm Cove meeting. The special issue of Human Mutation (Volume 23\#5, May 2004) contains a selection of interesting articles authored by speakers at the meeting in Palm Cove. This meeting report summarizes all of the presentations and, when appropriate, cites related articles from the issue. The special issue can be accessed online at the journal’s web site (www.wiley.com/humanmutation).

Since the first Mutation Detection meeting in 1991, the field has changed. In 1991, the focus was on how to detect and score rare, highly penetrant mutations to establish diagnostics for monogenic disorders. Today, while the importance of clinical diagnostics is recognized, a major focus of research on human genetic variation is to characterize the nucleotide sequence diversity on a genome-wide scale in all human populations. The Human Genome Project has been a major force driving this development. The Mutation Detection 2003 meeting was particularly happy to welcome one of the “first-timers” at this meeting series, namely Francis Collins (National Human Genome Research Institute [NIHGR], Bethesda, MD). Francis Collins gave an enthusiastic keynote lecture, describing the NIHGR’s vision for the future of genomic research [Collins, 2003], and presented some challenges to the meeting participants. One of the major challenges in genomics is to develop

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technology to bring down the cost of genotyping or nucleotide sequence analysis. Collins urged the scientists participating in the meeting who have an interest in technology development to further innovations toward a quantum shift in cost reduction.

**MUTATION SCANNING OR HIGH-THROUGHPUT SNP GENOTYPING?**

The talks and posters at the Mutation Detection 2003 meeting in Palm Cove featured both technology for identifying previously unknown mutations, i.e., mutation scanning or resequencing methods, and technology for scoring previously-known mutations or single nucleotide polymorphisms (SNPs) on a large scale. In clinical diagnostics, the same methods as those used for large-scale SNP genotyping are applied, for example, in the diagnosis of hemochromatosis and thrombophilia, and a number of monogenic recessive disorders caused by a relatively small number of mutant alleles. In other monogenic disorders, the mutant alleles are so rare that each family carries its own mutation, which is why routine clinical diagnostics must be based on mutation scanning over a complete gene. Examples of disorders requiring whole-gene scanning include familial cancers, thalassemias, and hemophilia. In Palm Cove, approaches taken by diagnostics laboratories were reviewed by Zbigniew Rudsky (Institute of Medical and Veterinary Science, Adelaide, Australia) and Andrew Laurie (Canterbury Health Laboratories, Christchurch, New Zealand).

The recently initiated haplotype mapping (HapMap) project aims to characterize the structure of sequence variation throughout the genome, to provide a guide for selecting SNP markers for mapping genes underlying common, multifactorial disorders. The HapMap project was described by Francis Collins, and was also touched upon by Mark Chee (Illumina, San Diego, CA), whose very high-throughput BeadArray™ technology will be one of the SNP genotyping systems used in the HapMap project.

Initially, for mapping genes underlying complex disorders, SNPs with common alleles such as those identified by the HapMap project will be genotyped. However, as pointed out by Raymond Miller (Washington University, St. Louis, MO), a large number of SNPs occur at low frequency in our genome. Many of the phenotypes observed in multifactorial traits may be caused by interactions between rare, population-specific, or even family-specific SNP alleles. Identification of these rare genetic variants will require large-scale resequencing of defined genomic regions in multiple individuals. Thus there is an urgent need to develop cost-effective methods both for large-scale SNP genotyping and mutation scanning.

**SPECIALIZED DATABASES**

All data from the HapMap project will be publicly accessible via the NCBI dbSNP database, and a unique intellectual property strategy is being developed to guarantee its accessibility. Some of the more specialized, ongoing database initiatives were alluded to at the meeting. Richard Cotton (Genome Variation Analysis Center, Melbourne, Australia) reviewed efforts to catalog all disease-causing mutations across the genome [Horaitis and Cotton, 2004]. The HUGO Mutation Database Initiative (MDI) was founded in 1994 and has evolved into the Human Genome Variation Society (HGVS; www.hgvs.org). The aims of the HGVS are to document and freely distribute information on all genetic variants associated with disease, and to provide guidelines on the mutation nomenclature. Kenshi Hayashi (Kyushu University Human Genome Centre, Fukuoka, Japan) described a database focusing on sequence variants in transcription start sites identified by single-strand conformation polymorphism (SSCP) screening. Paul Pearson (Transgenomic, www.transgenomic.com) described a database with genes containing rare sequence variants as well as denaturing high performance liquid chromatography (DHPLC) profiles for detecting them. Gary King (The University of New South Wales, Sydney, Australia) described an extensible markup language (XML) schema for data exchange—biological variant markup language (BVML)—and a database for combining information on biological variations at the level of DNA, mRNA, and protein.

**REACTION PRINCIPLES AND ASSAY FORMATS**

Perhaps surprisingly, the reaction principles underlying the methods presented at this meeting are largely the same as those discussed at the first Mutation Detection meeting over 10 years ago. Mutation scanning assays utilize similar physicochemical and mismatch cleavage methods as those of 10 years ago. SNP genotyping methods are based on nucleic acid hybridization with short oligonucleotide probes or on the use of DNA modifying enzymes, such as DNA polymerases, ligases, or nuclease. There has, however, been enormous development in assay formats and labeling and detection strategies. Miniaturization and multiplexing of the mutation scanning and genotyping assays is a key element for bringing down costs and increasing throughput. Two principally different approaches underlie the new miniaturized assays that offer the promise of cost reduction. Development of highly parallel assays in solid-phase microarray formats and homogenous assays performed in individual channels in microfluidic devices were addressed in several presentations.

**ADVANCES IN METHODS FOR MUTATION SCANNING**

DHPLC continues to be the workhorse for medium throughput mutation scanning. Recent developments in DHPLC were reviewed by Peter Oefner (Stanford Genome Center, Stanford, CA), the pioneer of DHPLC technology. For example, Oefner reported a new nonporous monolithic HPLC column matrix having an extremely long life-span. The advent of column arrays for
HPLCs could enable DHPLC to compete with the increased capacity of the multicapillary gel electrophoretic instruments that are widely used for large-scale sequencing, mutation detection, and SNP genotyping today. The two major suppliers of DHPLC instruments have each refined and extended their systems, as described by Paul Pearson (Transgenomic) and Eric Gerber (Varian, www.varianinc.com). Margaret Smith (Royal Melbourne Hospital, Melbourne, Australia) described the use of GC clamping on amplicons to simplify DHPLC assay design for the BRCA1 and BRCA2 genes.

Capillary electrophoresis systems for the other workhorse in mutation scanning, SSCP, were outlined by its inventor Kenshi Hayashi (Kyushu University Human Genome Centre, Fukuoka, Japan). In his center, SSCP analysis has been integrated into a laboratory information management system that also includes retrieval of SNPs from databases and interpretation of data. Paal Andersen (Statens Serum Institute, Copenhagen, Denmark) reported extremely high detection sensitivity when SSCP was used in a new capillary system. Also, the traditional melting gel techniques (temperature and denaturing gradient gel electrophoresis, TGGE and DGG) for mutation scanning are routinely performed with good success in capillary sequencing instruments, as reported by Thomas Peters (Ingenium Pharmaceuticals, Martinsried, Germany), Annette Torgunrud Kristensen (The Norwegian Hospital, Oslo, Norway), Carol Kosman (Applied Biosystems, Foster City, CA), and Volker Gurtler (Aurstin & Repatriation Center, Heidelberg, Germany). Maria Dulay (Stanford University, Stanford, CA) described free capillary electrophoresis for separating mismatched and matched peptide nucleic acid (PNA)/DNA hybrids.

Anthony Yueng (Fox Chase Cancer Centre, Philadelphia, PA) described the properties and action mechanism of a new nuclease extracted from celery (CEL I). The CEL I nuclease cleaves both DNA strands highly specifically at mismatches, and thus appears ideal for genomic mismatch scanning, as the cleavage products can be readily separated by capillary gel electrophoresis. Yueng showed convincing evidence of its use, including detection of a single mutation in a bacterial genome in 4 hr.

MICROARRAY-BASED GENOTYPING ASSAYS

Both hybridization and enzymatic genotyping methods are being used in microarray formats for genotyping and mutation scanning. The Affymetrix 10,000 feature gene chip, presented by Keith Jones (Affymetrix, Santa Clara, CA) uses allele-specific hybridization probes for typing a genome wide panel of 10,000 SNPs. This system can be used instead of microsatellite markers in genome-wide linkage studies. It may also be able to determine genome-wide gene dosage changes, offering an alternative to array-based comparative genomic hybridization (CGH) methods. Jones also outlined a modification of the system designed for genotyping custom-selected SNPs other than those in the predefined 10,000 feature panel.

Ligase is the key enzyme in Ulf Landegren’s (Uppsala University, Uppsala, Sweden) circularizable padlock probes, which have been used both for SNP detection in situ and for highly-parallel SNP genotyping in combination with universal Tag-arrays from Affymetrix. The latter “gap-fill” modification of Landegren’s padlock probes is also denoted “molecular inversion probes,” as featured by ParAllele Biosciences (Stanford, CA). Francis Barany (Cornell University, Ithaca, New York) addressed the diagnostic issues presented by tumor specimens, in which the sources of DNA for mutation detection are often a minority in a vast excess of wild-type sequences, by a combination of ExonucleaseV cleavage and ligase-mediated detection on a universal zip-code microarray. In her presentation, A-C. Syväs en (Uppsala University, Uppsala, Sweden) focused on quantitative SNP typing using “minisequencing,” which utilizes a DNA-polymerase for single-base extension followed by Tag-array capture in an “array-of-arrays” microarray format. In this assay format, up to 16,000 genotypes can be extracted from a single standard microscope slide. The same reaction principle is used in a 12-plex streamlined 384-well array format in the recently launched SNPstream system from Beckman Coulter (www.beckman.com). For mutation detection in cancer, Paolo Fortina (Department of Medicine, Thomas Jefferson University, Philadelphia, PA) uses Affymetrix Tag-array capture of single-nucleotide extension products and array-based resequencing mediated by the ligase chain reaction. The Code Link™ platform from Amersham Biosciences (www.amershambiosciences.com), presented by Carl Yamashiro, uses allele-specific primer extension in a microarray format for SNP typing. Joakim Lundeberg (Royal Institute of Technology, Stockholm, Sweden) uses an array-of-arrays format on microscope slides in his apyrase-mediated allele-specific extension assay. Both in routine diagnostics and large scale SNP mapping, array-of-array formats that allow analysis of multiple samples per microarray have obvious advantages over the “traditional” microarray formats designed for a single sample per slide.

MICROFLUIDIC DEVICES

Richard Mathies (University of California, Berkeley, CA) described a microscaled sequencing platform that integrates PCR, DNA cleanup, sequencing, and data analysis in a circular assay device, not unlike a compact disk. According to Mathies, this integrated device was able to process 96 simultaneous 300-nL sequencing reactions in 25 minutes. Another example of miniaturization was André Marszal’s (University of British Columbia, Vancouver, Canada) nanopore technology to detect single-base mismatches based on mismatch cleavage. Garry King (The University of New South Wales, Sydney, Australia) presented a new process for manufacturing miniaturized devices, and their application to both electrophoretic size-separation and
single-base extension assays. In this system, the extension products, combined with novel electrochemically tagged nucleotide substrates, or “electrotides,” can be directly detected on electrode surfaces [King et al., 2004]. Shigeori Takenaka (Kyushu University, Fukuoka, Japan) and Mashikio Amano (TUM Gene, Chiba, Japan) are developing a compact electrochemical array system. This method uses allele-specific hybridization probes in combination with an electrochemically active intercalating compound. Fully integrated miniaturized microfluidic devices are complex to manufacture, but technically simple to use. Thus mutation detection in microfluidic devices may find future applications in “point-of-care” diagnostics, provided that sufficient detection specificity and sensitivity can be achieved in these systems.

LABELING AND DETECTION STRATEGIES

Homogenous SNP genotyping assays are well suited for streamlined detection in microfluidic devices, but require detection methods that can distinguish between the labelled molecules that have become bound to the analyte and excessive unbound labelled molecules, of which fluorescence resonance energy transfer (FRET) is the most well-known principle. Tom Brown (Southampton University, Southampton, UK) reviewed the use of acridine-quenched fluorochromes (Hybeacons; LGC, www.lgc.co.uk) as a simple probe design compatible with rapid homogenous real-time PCR. A related stem/loop probe design used in a homogenous strand-displacement assay was presented by Wu Ming (Quantum Dot Corporation, Hayward, CA). Double-stranded hybridization probes were used in another homogeneous strand displacement assay presented by Zingge Li (Xiamen University, Xiamen, China).

Mass spectrometry has so far been used with good success for SNP typing by primer extension in the GOOD assay developed by Ivo Gut (Centre National de Genotypage, Evry, France) and in the MarrArray™ platform from Sequenom (San Diego, CA) [Gut, 2004]. According to Charles Cantor (Sequenom, San Diego, CA), mass spectrometry may be able to deliver resequencing of short fragments cleaved using either DNA glycosylases using the SNaPIT technology, as described by Pat Vaughan (HiberGren Ltd., Wicklow, Ireland), or by the novel rNTP/alkali cleavage method outlined by Ivo Gut. If successful, the capacity of the mass spectrometric platforms to deliver 30,000 genotypes per day could be adapted to 30,000 bases resequenced. This would have a profound effect on the cost and delivery of genetic diagnosis and identification of previously unknown SNPs.

SENSITIVE, QUANTITATIVE MUTATION DETECTION AND SNP GENOTYPING

To accomplish accurate and sensitive quantification of mutations and SNPs and to perform such analyses on a large scale is a technical challenge. Russell Higuchi (Roche Molecular systems, Alameda, CA), described the application of quantitative SNP genotyping in pooled samples for increasing throughput in large-scale association studies. The genotyping method used is based on multiplex allele-specific real-time PCR followed by quantitative detection of the PCR product by “reverse dot blot” hybridization to probes immobilized nylon strips. Yoichi Matsubara and Shigeo Kure (Tohoku University School of Medicine, Sendai, Japan) described a closely related method with detection using an immunochromatographic strip.

In general, a major advantage of allele-specific PCR is its ability to detect minority mutations, but accuracy and sensitivity are limited by the error rate of the DNA polymerase. By combining allele-specific PCR and serial coupling of pyrophosphorolysis, Liu Chiang Liu and Steve Sommer (City of Hope/Beckman Research Institute, Duarte, CA) succeeded in improving the specificity of primer extension and reported detection of one mutant SNP allele in the presence of up to $1 \times 10^6$ copies of wild-type sequence in a lambda phage model system [Liu and Sommer, 2004].

Mike Makrigiorgos (Dana Farber–Harvard Medical School, Boston, MA) described the use of the mismatch repair enzymes combined with chemical cleavage to detect low-level mutations [Makrigiorgos, 2004]. Using a recent development of the mismatch repair assay based on restriction enzyme cleavage and hairpin PCR, they have been able to achieve a detection sensitivity of one mutant SNP allele from an excess of $1 \times 10^6$ wild-type alleles.

Quantification of gene dosage differences, caused by genomic deletions or insertions of varying size, using multiplex amplifiable probe hybridization (MAPH) and multiplex ligation-dependent probe amplification (MLPA) were reviewed by Loryn Sellner (Princess Margaret Hospital, Perth, Australia) and Graham Taylor (Regional Genetics and Cancer Research UK Laboratory, St James’s University Hospital, Leeds, UK). Both methods rely on exon-specific hybridization to genomic DNA, followed by amplification and quantification of the amplified probes. MAPH and MLPA are relatively new approaches that were featured in several of the posters, and MLPA, in particular, seems to be gaining widespread use in the diagnostic community [Sellner and Taylor, 2004].

FUTURE CHALLENGE I: AVOIDING PCR AS RATE LIMITING STEP

The PCR technique provides both the sensitivity and specificity required for detecting single nucleotide variations in large complex genomes, such as the human genome. Today, while assay formats and detection technologies have developed tremendously, the difficulty of performing multiplex PCR has become rate-limiting in most systems for high-throughput genotyping. However, solutions to this problem are emerging.

Yasuyuki Ikeda (National Cardiovascular Institute, Osaka, Japan) demonstrated that the Invader assay was
able to reliably screen the lipoprotein lipase gene for known mutations in genomic DNA from patient samples without the need for PCR amplification. In the Invader assay, sufficient specificity is accomplished by two hybridization probes that recognize the target sequence simultaneously, in combination with endonuclease cleavage of one of the probe in an allele specific manner. Sensitivity is achieved by performing a secondary Invader assay with the cleaved probe as target. In the Illumina BeadArray™ system, described by Mark Chee, and in the Molecular inversion or Padlock probe approaches, described by Ulf Landegren, sufficient specificity for genotyping over a thousand of SNPs directly in genomic DNA is achieved by simultaneous hybridization of two ligation probes to genomic DNA, followed by “gap fill” primer extension of one of the probes and joining of the probes by ligation. In both systems, PCR with universal primers is finally used as a means for signal amplification to obtain sufficient sensitivity of detection. These two methods will serve as platform technologies in the HapMap project, and in the near future we will see data on the quality of the genotypes produced.

In the Affymetrix 10K GeneChip system, described by Keith Jones, the complexity of the genome is reduced to a level that allows SNP genotyping by restriction enzyme digestion, linker ligation, and PCR with universal linker primers, including a size-selection step. Andrezej Kilian (Cambia, Canberra, Australia), reported whole-genome mapping in plants using a related scheme for complexity reduction.

**FUTURE CHALLENGE II: EXPERIMENTAL HAPLOTYPING**

Today, genotyping of SNPs in association studies is usually performed without information on phase of inheritance of physically closely related SNP alleles. To assign SNP haplotypes experimentally, genotyping in three-generation families or cloning of individual chromosomes are required. Ivo Gut presented a scheme based on multiple allele-specific PCRs for experimental haplotyping. Although his scheme is useful in some situations, it is limited to haplotypes formed by SNPs that are located close enough to each other to be amplified within the same PCR fragment. An exciting and futuristic approach toward experimental haplotyping was presented by Pui-Yan Kwok (University of California, San Francisco, CA) [Kwok and Xiao, 2004]. Together with a group specializing in nanotechnology, Kwok is developing an approach where the SNP haplotypes are assigned in single DNA molecules labeled allele-specifically with combinations of different fluorescent probes. To score the combination of fluorophores corresponding to the haplotypes on individual molecules, a microfluidic system that allows separation and detection of individual, long DNA molecules is required. This is evidently a demanding task.

**CONCLUSION**

With the landmark sequencing of the human genome behind us, we now need to understand the range and significance of genomic sequence variation. *Mutation Detection* 2003 focused minds on current developments and also highlighted areas in which further developments are needed. Francis Collins included the improved synthesis of long DNA molecules, an encyclopedia of DNA elements and their functions, and a comprehensive survey of DNA methylation in his wish list of tools for genomic medicine. These tools would directly impact the field of mutation detection and genotyping. Collins also predicted that many of the major genes contributing to diabetes, heart disease, cancer, mental illness, Alzheimer disease, Parkinson disease, asthma and other complex disorders, will be identified within the next 5 to 10 years. Perhaps then these disorders will be targets for specific DNA-based diagnostics, analogous to the monogenic disorders today. Collins concluded his lecture by commenting that, in order for all populations to benefit fully from genomic medicine, all populations need to be part of genomic research. In organizing meetings like this one, HUGO is making an important contribution to that process.

**REFERENCES**


