Rapid, inexpensive scanning for all possible BRCA1 and BRCA2 gene sequence variants in a single assay: implications for genetic testing

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Recent advances in functional genomics have fostered the vision of a future health care that will be tailored to the specific needs of a person by using detailed genetic information about disease predisposition, disease progression, and the response to preventive or therapeutic medication. To confirm that this concept of personalised medicine is viable, proof of principle studies are required, entailing extensive efforts to identify and functionally characterise individual gene variants in human populations and their association with disease or therapy related phenotypes. For this purpose, as well as for the ultimate widespread genetic testing that may result from these studies, practical, cost efficient methods of analysing thousands of genes in large human populations for all possible sequence variants are critically important. Such methods are currently not available, the main stumbling block being the high costs associated with comprehensive genotyping of large numbers of DNA samples. Costs are determined to some extent by intellectual property rights on the genes and gene variants (in fee for service testing), but mostly by the type of assay that is used. When a genetic test can be based on one or few mutations, the costs are generally low, owing to the recent increase in assays capable of confirming the presence or absence of a previously known mutation or single nucleotide polymorphism (SNP). Unfortunately, even relatively simple monogenic diseases, such as cystic fibrosis, can be caused by many different mutations in the same gene and it will never be possible to test only for a limited number of variants.

The situation is different for the genetic component of complex diseases. The question here is if such diseases involve combinations of a few common SNP haplotypes or many rare alleles. Recent results indicate that most gene variants occur at low frequency, which suggests that association or family studies on the basis of a limited number of common SNPs in candidate genes would not be the optimal strategy to find all the relevant variants that could serve to guide future management of such diseases. Instead, it might be necessary exhaustively to interrogate the entire coding and regulatory regions of many candidate genes in large populations to ensure discovery of all relevant variants, including the ones that may be found associated with a disease or therapeutic phenotype only in some but not all populations. When all clinically relevant variants of a gene are identified the number may be so large that, like in the discovery phase, resequencing might still be the most practical option, also in the clinical setting. Hence, there is a need for comprehensive screening methods that are not limited to a given number of common gene sequence variants and that can be applied cost effectively to many different genes.

At present, nucleotide sequencing is the gold standard in genetic testing, as exemplified by the BRCA1/BRCA2 genetic test for breast and ovarian cancer offered by Myriad Genetics for $2800. While this price tag is generally considered high, it is doubtful that even on a non-for profit basis a significant reduction is feasible, for example, by 10-100-fold. To address this problem, several alternative methods for comprehensive genotyping of subjects have been developed. Most of these methods suffer from a lack of either accuracy or applicability in clinical routine. Virtually all of them are based, like sequencing, on the PCR amplification of individual target sequences (that is, exons and their surrounding intronic regions) followed by their serial analysis for sequence variation. An exception is the use of microarrays, pioneered by Affymetrix, to interrogate each nucleotide position in a gene, using PCR amplified coding regions as probe. At present, this method is not yet fully developed and still too costly.

A mutation detection principle with both high accuracy and the potential to be developed into a high throughput screening system at low cost is denaturing gradient gel electrophoresis (DGGE). It has been convincingly shown that DGGE has the same or higher sensitivity as nucleotide sequencing and has been converted into a system capable of assessing multiple fragments in parallel using a combination of multiplex...
PCR and two dimensional electrophoresis. Termed two dimensional gene scanning (TDGS), this method has now been shown to be useful in screening a large variety of genes for all possible sequence variation, including some major disease genes such as BRCA1, TP53, and MLH1. In a recent comparison with three other assays for detecting unknown mutations in BRCA1, that is, single strand conformational polymorphism analysis (SSCP), conformation sensitive gel electrophoresis (CSGE), and denaturing high performance liquid chromatography (DHPLC), TDGS and DHPLC performed best with a 91 and 100% detection rate, respectively.

Figure 1  Two dimensional gene variant scanning of BRCA1 and BRCA2 in a single assay. (A) Complete separation of 115 fragments corresponding to the entire coding regions of BRCA1 and BRCA2. Primers for each TDGS fragment were designed according to optimal melting behaviour, with a GC clamp attached to one primer of each pair. Primers were fluorescently labelled with HEX (green spots, representing 40 BRCA1 fragments), FAM (blue spots, representing 25 BRCA2 fragments), and Texas Red (red spots, representing 50 BRCA2 fragments). To increase specificity, PCR amplification was performed in two steps. First, three long distance multiplex PCRs were performed to amplify 14 large fragments encompassing the coding regions of the BRCA1 and BRCA2 genes. These products served as template for 12 multiplex short PCRs to amplify all 115 target fragments. One µl of each multiplex short PCR was combined (12 µl total) and loaded onto a 0.25 mm polyacrylamide denaturing gradient gel (0.75% urea/formamide). Separation according to size and base pair sequence (melting temperature) was for three hours and 27 minutes. The entire gel cassette was then scanned using an FMBIO II Fluorescent Image Scanning Unit. (B) Separate TDGS pattern for 40 HEX labelled BRCA1 fragments. Sequence variants were detected in exons 11.6, 11.7, 11.8, 11.9A, 11.11, 11.13, 11.14, 13, 16, and 24. (C) Separate visualisation of the TDGS pattern for 25 FAM labelled BRCA2 fragments. A sequence variant was detected in exon 10.3. (D) Separate TDGS pattern for 50 Texas Red labelled BRCA2 fragments.
DHPLC is based on the same denaturing gradient principle as TDGS, but has a much lower throughput since it operates on a fragment by fragment basis, making interpretation of the results easier. So far, TDGS has only been used in the research setting, but with the development of an instrument for automated 2D electrophoresis and the use of multicolour fluorescent labels high throughput applications in the routine setting have become feasible. Here we show the utility of a single, low cost TDGS test as an objective screen for all possible mutations and SNPs in the BRCA1 and BRCA2 coding regions.

METHODS AND RESULTS

Using PCR amplification in 12 multiplex groups with three different fluorophores, all 115 gene coding fragments were obtained and subsequently subjected to automatic 2D denaturing gradient gel electrophoresis (procedures are briefly summarised in the legend to fig 1 and provided in detail on http://fgoa.stcbmlab.uthscsa.edu/supplemental/AGC-BRCA1+2/). After fluorescent scanning of the gel, the complete three colour pattern was first visualised on the screen (fig 1A). The pattern of each colour group was then interpreted separately by eye directly from the screen and sequence variants (in brackets) were scored electronically (fig 1B-D). As described previously, it is possible to differentiate between common SNP variants or recurrent mutations and new or rare sequence variants on the basis of the unique spot configuration of each heterozygous variant. Variant fragments for which the spot configuration indicated a new mutation or SNP are subjected to confirmatory testing using nucleotide sequencing.

DISCUSSION

In view of its low cost and simplicity, TDGS has the potential of becoming a method of choice for large scale genetic epidemiological research until more advanced sequencing methods currently under development become available. Based on the empirically determined cost of about $50 for screening the 115 fragments of the large BRCA1 and BRCA2 genes on one gel (see http://fgoa.stcbmlab.uthscsa.edu/supplemental/AGC-BRCA1+2/ for details), the costs of analysing a gene with an average coding region of about 2000 bp (corresponding to 20 or less TDGS fragments) would be only about $10. This would permit population studies of significant sample size and number of TDGS fragments) would be only about $10. This would permit population studies of significant sample size and number of TDGS fragments) would be only about $10. This would permit population studies of significant sample size and number of TDGS fragments) would be only about $10. 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