PCR–SSCP–HDX analysis of pooled DNA for more rapid detection of germline mutations in large genes. The BRCA1 example

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Several reports from laboratories involved in the isolation of the BRCA1 gene, and recently in the search for its mutations (1–5), show that the latter activity is not a simple task. Every fragment of coding sequence of this multi-exon gene has to be analysed for scattered germline mutations taking BRCA1 as an example. Usually all BRCA1 exons, together with flanking intron sequences or cDNA fragments, are sequenced directly. It is, however, labour intensive. Alternatively, they are first screened by single strand conformation polymorphism (SSCP) (7) to increase throughput of analysis. In the case of BRCA1 ~50 different gene fragments are analysed by SSCP and reamplified pure mutant species are sequenced. It is also our experience (J. Czarny et al., in preparation), that this approach becomes very laborious, if a large number of chromosomes is to be tested. As no more reliable technology for large scale mutation screening is at hand now, we took some measures to increase the throughput of SSCP analysis without compromising its ability to detect mutations. Assuming that the frequency of BRCA1 mutations is 0.5% in the general population (3) screening of 200 individuals would give, on average, a single mutation. Detecting this mutation would require running 200 SSCP gels (one gene fragment from 50 individuals on one gel and 50 gene fragments analysed). The question we addressed in this study was how to cut this number by a factor of 10 or more.

First, we have analysed the possibility of doing PCR–SSCP assay on genomic DNA pools of various size. The optimum number of individual DNA samples to include in a pool is determined by the ability of detecting variant SSCP bands by autoradiography. To test the limits of pooling, the genomic DNA samples from different individuals were mixed together and subjected to PCR followed by SSCP analysis which is in fact the SSCP–heteroduplex (HDX) analysis in the conditions we use. For these experiments we have included the genomic DNA from patients in which mutations or polymorphisms in BRCA1 were earlier found and carefully characterised (J. Czarny et al., in preparation). It turned out that in pools composed of four to five DNA samples, fragments containing germline mutations could be easily detected.

In the case of germline mutations, one copy of the gene has a wild-type sequence and the other contains a mutation. According to our experience this fraction of mutant DNA, when present in a variant SSCP band, can be detected even when diluted 40 times with the signal from the wild-type sequence. However, in conditions of SSCP analysis, one or both DNA strands very often form not a single but two or more unevenly represented stable conformers showing different mobility. In the worst case, a new conformer created by a mutation may contribute 5% or even less to the overall radioactivity signal. To detect such less prominent signals, the number of DNA samples combined in a pool should not be higher than four to five. Otherwise they are barely discernible and could be missed. There are also other examples where mutation does not change the gel migration of any of the single strand conformers but the mobility of the heteroduplex formed is different. This is sometimes observed in cases of very small insertion and deletion mutations. The influence of DNA pooling on the ability of detecting these less prominent variant bands is shown in Figure 1.

Next, we asked the question which fragments of the gene could be analysed together on the same gel in a multiplex version of SSCP. In order to demonstrate this clearly, we have run all 49 different fragments of BRCA1, including 26 fragments of exon 11, on a single native polyacrylamide gel. It turned out that SSCP analysis of BRCA1 can be done on 10–12 pools, each composed of four to five different gene fragments, despite the fact that BRCA1 primers were not specifically designed to facilitate the multiplex type of analysis. The example of multiplex SSCP–HDX analysis of six different BRCA1 exons is shown in Figure 2.

Next, the approach outlined above was compared directly with the standard procedure in the real mutation screening. This trial involved genomic DNA from 96 individuals and 23 BRCA1 exons. The same genomic DNA samples were subjected to PCR, both separately and in pools of four. Then the DNA fragments amplified from pooled DNA were combined into groups of four before the SSCP–HDX analysis. Except for longer autoradiography of the gels with pooled DNA samples, other conditions of analysis were the same. It turned out that each of the nine variant bands that were detected by the conventional type of analysis were also detected in pooled DNA.

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amplifications are conducted in a 5 µl volume following the protocol for fast and economical PCR (9). When a pool of five genomic DNA samples is amplified in the 5 µl volume it is like using 1 µl PCR mix to amplify a gene fragment from a single genomic DNA sample. Further reduction of time and effort required to detect mutations could be achieved by using fluorescently labeled primers and automatic DNA sequencing apparatus for SSCP–HDX analysis (10). The replacement of a single radioisotope with four different fluorescent dyes should enable a further increase of throughput of the analysis and enhance the sensitivity of detecting mutations.

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