OPTIMIZATION OF HETERODUPLEX ANALYSIS FOR THE DETECTION OF *BRCA* MUTATIONS AND SNPs

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Abstract: *BRCA1* and *BRCA2* are tumour suppressor genes whose mutant phenotypes predispose to breast and ovarian cancer. Screening for mutations in these genes is now standard practice for hereditary breast and ovarian cancer (HBOC) cases in Europe, and permits medical follow-up and genetic counselling adapted to the needs of individuals in such families. Currently, most laboratories performing diagnostic analysis of the *BRCA* genes use PCR of exons and intron-exon boundaries coupled to a pre-screening step to identify anomalous amplicons. The techniques employed for the detection of mutations and SNPs have evolved over time and vary in sensitivity, specificity and cost-effectiveness. As a variant for pre-screening techniques, we chose the recently developed Surveyor® heteroduplex cleavage method as a sensitive and specific technique to reveal anomalous amplicons of the *BRCA* genes, using only basic laboratory equipment and agarose gel electrophoresis. Here we present the detection of either mutations or SNPs within the *BRCA1* exon 7, using heteroduplex analysis (HA) by mismatch-specific endonuclease, confirmed by dideoxy sequencing.

INTRODUCTION

Hereditary breast and ovarian cancers are mainly attributable to predisposition genes whose germinal mutations predispose to the disease [Antoniou et al., 2003]. As both pathologies are common in women of western world [Ferlay et al., 2000], identification of such predisposition genes generated an over 15 years candidate-finding race in cancer research. Since the identification of the main breast/ovarian cancer genes, *BRCA1* in 1994 [Miki et al., 1994] and BRCA2 in 1995 [Wooster et al., 1995], a dozen of additional candidates have been reported, some of them being already included in diagnosis tests [Narod et al., 2004]. However, the *BRCAs* are the principal responsible in over 1/3 of hereditary cases, that mean familial agglomerations with 2 or more early onset breast or/and ovarian cancers. Screening for mutations in these genes is now standard practice for hereditary breast and ovarian cancer (HBOC) cases in Europe, and permits medical follow-up and genetic counselling adapted to the needs of individuals in such families. Several problems appear when investigating the *BRCA* status for an individual, with or without a family history.

Firstly, the two genes are very large [NCBI Genbank]. *BRCA1* is composed of 5592 nucleotides and contains 24 exons sparing 100 kb genomic DNA. *BRCA2* is even larger, 10257 nucleotides containing 27 coding exons and sparing 70 kb DNA. As an additional difficulty, both genes possess an unusually large exon 11, of 3,4 kb in *BRCA1* and 5 kb in *BRCA2*. Even though limiting sequencing to exonic regions and exon/intron boundaries, there is a huge amount of work to do when attempting to completely investigate *BRCA1* and *BRCA2*. That will comprise a total of 84 amplicons to be forward and reverse sequenced, which means a very expensive and time consuming approach.

Secondly, *BRCA* mutational spectrum has not been entirely characterized, and not all sequence variants are clearly pathogenic. Over one thousand small sequence variations have been reported by now in the Breast Cancer Information Core (BIC) database. More than half of these mutations cause the loss of function by premature protein synthesis termination, and around 60 % are unique to a family. Other variations include mis-sense alterations and intronic variants with unknown disease relevance. These are classified as benign polymorphisms or unclassified variants (UV) with unknown pathological potential. To date, 43.5 % of *BRCA* variants are of uncertain clinical significance.

Nonetheless, cancer predisposition diagnosis is a hard to assume and delicate problem to manage, so all the resources and means are never sufficient to avoid the mistake. False positives and false negatives are always pending behind, and several different techniques are absolutely necessary in order to bring the error close to null. Besides iterative verifications of a positive result, one should always combine diverse techniques available, and generate a multilevel validated result. Therefore during the last few years, the whole molecular biology scientific community starter a large campaign of searching and comparison of alternative pre-screening methods, with the aim of early identification of mutation bearing susceptible amplicons which will be sequenced. The techniques employed for the detection of mutations and polymorphisms have evolved over time and vary in sensitivity, specificity and cost-effectiveness. The protein truncation test (PTT) has largely been set aside due to low sensitivity and specificity or specificity that is too narrow [Hogervorst et al., 1995]. Currently, most laboratories in Europe performing diagnostic analysis of the *BRCA* genes use PCR of exons and intron-exon boundaries coupled to a pre-screening step to identify anomalous amplicons [Negura et al., 2006]. Denaturing gradient gel electrophoresis (DGGE) or single strand conformation analysis (SSCP) can be used, both of them limited by great number of false positives and difficulties in interpretation of some polymorphisms. Alternatively,

denaturing high pressure liquid chromatography (DHPLC) presents the disadvantage of requiring either repeated analysis using an array of conditions and/or expensive reagents.

Assuming that direct sequencing is too expensive to apply systematically to all HBOC and sporadic cases, and that reliable DGGE, SSCP and DHPLC also require either repeated analysis using an array of conditions and/or expensive, specialized equipment and consumables, we turned our attention to the recently developed Surveyor® heteroduplex cleavage method (*Transgenomic*™) as a sensitive and specific technique to reveal anomalous amplicons of the *BRCA* genes using only basic laboratory equipment. Endonuclease cleavage of mismatch-containing DNA duplexes is a classic method for the detection of mutations and polymorphisms. Unfortunately, the enzymes available to carry out such experiments had widely different efficiencies for cleavage of the different possible mismatches and for the two DNA strands. A newly commercialized mismatch endonuclease isolated from celery, Surveyor® has been shown to be efficient at recognising all possible DNA mispairs as well as bubbles due to insertions or deletions, and to cleave both DNA strands 3' to the unpaired region. Since its first commercialization, Surveyor® enzyme has been successfully used in numerous applications, including mitochondrial DNA mutations analysis [Bannwarth et al., 2006) or SNP genotyping [Mitani et al., 2006]. This enzyme has been used to detect mutations in tumor DNA samples [Janne et al., 2006] using DHPLC to detect cleavage products, though many strategies for the detection of cleavage products are possible.

We implemented heteroduplex analysis with Surveyor® endonuclease, using a peripheral blood DNA protocol, in order of pre-screening *BRCA* mutations. Here we present the detection of either mutations or SNPs within the *BRCA1* exon 7.

MATERIALS AND METHODS

We recruited either HBOC families with three or more breast or ovarian cancer cases within the same family line, or sporadic breast and ovarian cancer cases. All patients agreed by written informed consent. Genomic DNA was extracted from 10 ml peripheral blood by using Wizard® Genomic DNA purification kit (*Promega*™). DNA amount was estimated by spectrophotometry. Polymerase chain reaction amplifying *BRCA* exon 7 was performed in a final volume of 50 µl containing 100 ng genomic DNA, 0.2mM each dNTP, 1,5 mM $MgCl_2$, 20 pmoles of each primer (sequence available on demand), and 0,25 units of GoTaq® Flexi DNA Ploymerase (*Promega*™) in 1X adequate buffer. PCR program was optimized is several steps to generate specific and efficient amplification, as described below.

Formation of heteroduplex molecules and their cleavage by Surveyor® were performed as suggested by the manufacturer (*Transgenomic*™), as explained below, and analysed after electrophoretic separation on agarose gels.

For DNA sequencing, amplicons were verified by electrophoresis on a 1,3% agarose gel, then purified by ExoSap® enzymatic digestion (*Affymetrix*™), following producer's instructions. The product was sequenced in forward and reverse reactions, using the BidDye® Terminator Cycle Sequencing Kit (*Applied Biosystems*™), according to the manufacturer's instructions. Cycle sequencing consisted of an initial denaturation step at 94ºC for 11 min, followed by 30 cycles of 94ºC for 10 sec, 52ºC for 5 sec and 30ºC for 3 min. Sequence analysis was performed using the Seqman® (*DNA Star Inc*™) software. Mutation presence was systematically confirmed by forward and reverse sequencing on a second independent blood sample.

Mutations and sequence variants are described according to HUGO approved systematic nomenclature. The nomenclature for BIC traditional mutations is also indicated.

RESULTS AND DISCUSSION

Heteroduplex analysis by mismatch-specific endonuclease supposes performing the following steps (Figure 1):

1) PCR amplification of the region of interest for all investigated patients (and eventually for references if needed);
2) generation of heteroduplex molecules by denaturation / renaturation of heterogeneous amplicons;
3) cleavage of heteroduplex molecules by the Surveyor® mismatch-specific endonuclease;
4) visualization of digestion fragments by electrophoresis in agarose gel stained with ethidium bromide.

Cleavage of a heterodulpex molecule by the Surveyor® endonuclease indicate the presence of a mismatch (generally, of a impairing due to nucleotide change, or bubbles due to insertions or

deletions), which means heterogeneity of investigated DNA. Since *BRCA* mutations are always heterozygous, the DNA of a carrier will always be heterogenous. In the case of other homozygous sequence variations like common SNPs, heterogenous DNA can be obtained by mixing patient DNA with reference DNA known as wild-type homozygous.

When using heteroduplex analysis as pre-screening method, ideally only one amplicon need be sequenced: the one containing a disease-causing mutation. In practice, samples heterozygous for a polymorphism must be sequenced because of the inability using Surveyor® alone to distinguish between a known polymorphism and a mutation giving rise to similar cleavage products. There is a concrete need for detecting deleterious mutation in diagnosis; there also is a less urgent need for detecting unclassified variants and SNPs for understanding cancer genetics. We tried to imagine a dual system able to identify both mutations and SNPs, and we present an example below for *BRCA1* exon 7.

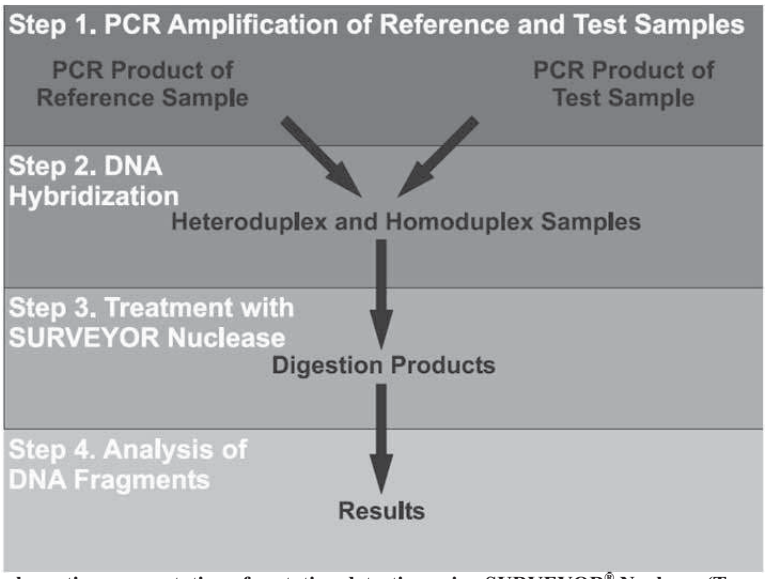

Figure 1. A schematic representation of mutation detection using SURVEYOR® Nuclease (Transgenomic™)

In figure 2 one can observe 325 bp bands corresponding to *BRCA1* exon 7 amplification of genomic DNA from different patients. Following optimization, amplification comprised an initial denaturation step at 94ºC for 5 min followed by 15 cycles of denaturation – 94ºC for 20 sec, annealing – 60ºC for 10 sec and extension – 72ºC for 15 sec, followed again by 25 cycles of denaturation – 94ºC for 20 sec, annealing – 56ºC for 10 sec and extension – 72ºC for 15 sec, and a final extension of 10 min at 72ºC. This touch-down type program is used in order to avoid unspecific product generation that we observed at the beginning. As we can notice in figure 2, amplification after optimization is efficient and specific. Lane 8 represents the no template control, which verifies absence of any contamination.

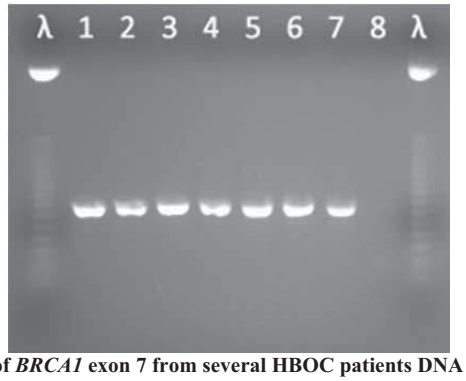

Figure 2. Amplification of *BRCA1* exon 7 from several HBOC patients DNA (λ = 50 bp step ladder).

In figure 3 are represented the results for heteroduplex analysis corresponding to exon 7 amplifications. Concretely, after verification in gel, amplicons were treated by a progressive denaturation / renaturation program (95ºC for 2 minutes, followed by cooling at 2ºC/second until 85ºC, then cooling at 0,1ºC/second until 25ºC). 10 µl of such obtained molecules were treated 20 minutes with 1,25 µl Surveyor® endonuclease (*Transgenomic*™), in the presence of 1,25 µl enhancer solution, following producer's instructions. By optimization of this digestion protocol, we obtained a better resolution for all bands, as is shown in figure 3b comparatively to bands observed in figure 3a. Optimization changes were cooling at 1ºC/second from 95ºC until 85ºC, and endonuclease treatment extended to 30 minutes.

In figure 3a, lane 13 corresponds to an undigested heteroduplex product, while digested molecules were migrated in lanes 1-11. Lane 12 represents the no template control, which verifies absence of any contamination. For three of our patients (lanes 1-3), two digestion bands can be observed around 150 and 180 bp. Those bands are certainly less intense then the undigested 325 bp product, which cannot be digested totally as patients are heterozygous for any deleterious variant. In figure 3b, those heterozygous patients are migrated in lanes 3-5, and we can observe a better resolution due to the diminution of undigested product intensity. That means a better digestion efficiency.

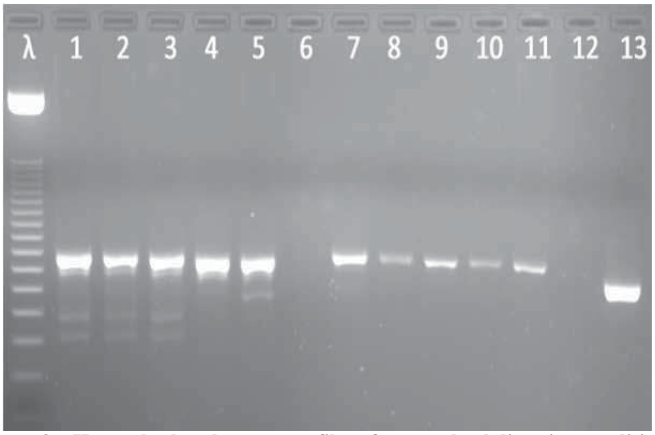

Figure 3a. Heteroduplex cleavage profiles after standard digestion conditions

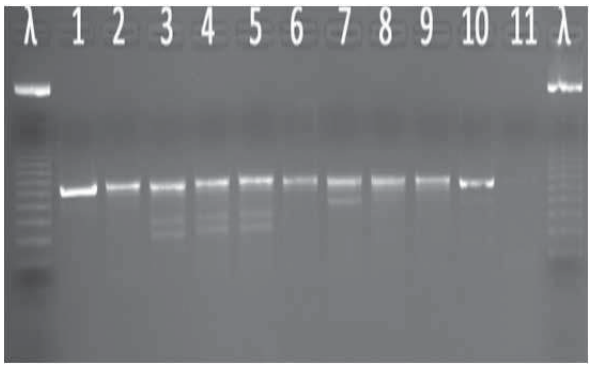

Figure 3b. Heteroduplex cleavage profiles after optimization of digestion conditions

When sequencing the concerned amplicon (figure 4), we detected a deleterious mutation 140 bp from the 3’end. In a heterogenous heterozygote DNA, heteroduplex molecules are formed at this level, and the Surveyor® endonuclease splits the 325 bp amplicon in two distinct fragments of 140 and 183 bp, on the mutant allele. Two nucleotides are missing, as the mutation is a 2-nucleotide deletion called c.342_343delTC in HUGO nomenclature (or 461delTC in the BIC nomenclature). We already reported [Negura et al., 2010a] this rare mutation causing a TGA Stop codon apparition and the premature termination of the protein synthesis after 114 N-terminal aminoacids (p.Pro115Stop). Patients in lanes 1,2,3 in figure 3a and lanes 4,5,6 in figure 3b are in consequence heterozygous for BRCA1 c.342_343delTC, as demonstrated by heteroduplex analysis confirmed by direct sequencing. Patients in lanes 4,7,8,9,10,11 in figure 3a and lanes 2,6,8,9,10 in figure 3b are wild-type homozygous, as no heteroduplex was cleaved by mismatch-specific nuclease. Undigested control product is lane 13 in figure 3a and lane 1 in figure 3b. No template control is lane 12 in figure 3a and lane 11 in figure 3b.

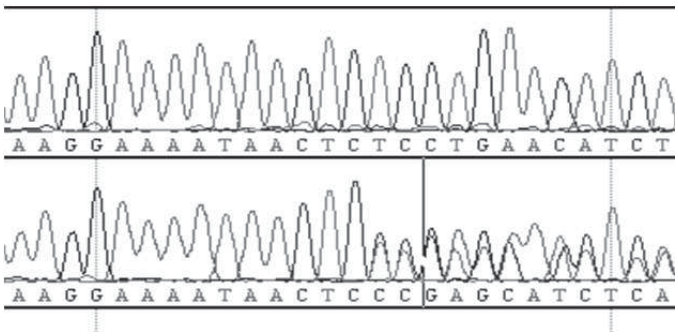

Figure 3b. DNA sequencing detects the c.342_343delTC mutation (down), as heterozygous sequence compared to wil-type homozugous (up)

Patient in lane 5 (figure 3a) or 7 (figure 3b) shows a different digestion profile, with an additional band around 240 bp. In fact, this patient is heterozygous for an unclassified sequence variant within exon 7, called c.427G>C in HUGO nomenclature, or 546G>C in BIC nomenclature. This apparent non-pathogenic UV sequence was described elsewhere [Negura et al., 2010b], and generates a mismatch 226 nucleotides from 5’end, so the nuclease cuts the 325 bp amplicon in a 226 bp (visible) fragment and a 99 bp (non-detectable) fragment.

CONCLUSIONS

Our results prove the feasibility of a rapid inexpensive pre-screening by heteroduplex analysis, in order to identify abnormal *BRCA* amplicons to sequence. As preliminary technique, HA by mismatch-specific endonuclease shows enough sensitivity to detect either deleterious mutations or various benign sequence alterations (SNPs, UVs). When present in a heterozygous context, allelic variations can be detected directly on heterogenous sample DNA; otherwise, an additional step is needed for mixing sample DNA with wild-type reference DNA.

We recommend therefore optimized Surveyor® heteroduplex as good pre-screenig *BRCA* method which greatly reduces the number of amplicons requiring sequencing.

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