MUTATION ANALYSIS IN FAMILIAL BREAST CANCER PATIENTS

Erik Teugeles, Jacques De Grève

Free University of Brussels

Abstract: Using a combination of three complementary screening techniques we investigated 186 Slovenian families with 2 or more cases of breast/ovarian cancer and found a cancer predisposing mutation in 57 families (30%). When considering families with more than 4 breast cancer cases or with at least one ovarian tumour, the mutation recovery rate rises to 50%. The BRCA1/2 mutations found in the Slovene population are distributed over the whole length of both genes but because of the high incidence of a few of these mutations, a restricted molecular screening covering only four DNA fragments can identify two third of them. A limited screen can thus be offered to persons that do not meet the strict intake criteria. Finally, since the genetic cancer predisposing factor seems to be missed in a significant fraction of the families (50% of Slovenian families), efforts are made worldwide (1) to ameliorate the screening efficiency of the BRCA1/2 genes and (2) to identify other breast cancer predisposing genes.

Breast cancer is a very common disease affecting some 10% of western women. Although the disease occurs mostly in the sporadic form, familial clustering is observed in about 10% of the cases. Linkage analyses performed in the early nineties, aimed to identify the chromosomal regions involved in this genetic cancer predisposition, indicated that 2 loci located at 17q21 and 13q12 must be responsible for the disease in 80% of high risk families. Two breast cancer predisposing genes, BRCA1 and BRCA2, were identified respectively in 1994 and 1995. From that moment on, molecular analyses of the BRCA1 and BRCA2 genes became possible, and knowing the disease causing mutation in a particular family became a very important tool for the counselling of this family.

BRCA1 and BRCA2 are very large genes organized in respectively 23 and 27 exons. Moreover, it became rapidly clear that the identified mutations are distributed over the whole length of the coding sequences. Mutation screening thus appeared to be a time and money consuming enterprise and the need for selective intake criteria was obvious. Today, mutation analysis of the full coding sequence of BRCA1 and BRCA2 can be offered by the majority of the genetic diagnostic laboratories, although the used screening techniques may vary somehow.

In our laboratory (VUB Brussels) we use a combination of 3 different techniques to screen the BRCA1/2 genes. The analyses are performed on genomic DNA prepared from blood samples of cancer affected persons.

The Protein Truncation Test (PTT) is applied for the analysis of the few large exons. Genomic stretches of DNA obtained by PCR are used IN VITRO to generate the corresponding protein sequences. These proteins are subsequently analysed on a polyacrylamide gel. A control person (with wild type DNA sequences) will generate a protein with a specific length. However, a patient carrying a nonsense or frameshift mutation in one allele will in addition show a second band on a protein gel, the smallest one corresponding to the mutant form. The advantage of the PTT technique is that it is less time consuming, can be applied on relatively large PCR fragments (and thus convenient for the analysis of large exons), and all identified mutations can be classified as cancer predisposing since they generate truncated proteins. Disadvantage is that we miss the missense mutations. However, this is not seen as very disturbing since only very few missense mutations can be classified as cancer predisposing at this moment. The majority of them are benign polymorphisms or are reported as "unclassified variants" and thus non-informative for clinical purposes.

The Denaturing Gradient Gel Electrophoresis (DGGE) is used to analyse all the remaining protein encoding small exons (57 in total). PCR fragments are first generated for each of these exons. The fragments are then heat denatured and renatured. When performed on a control sample (both alleles being identical and wild type), this will generate only one type of double stranded DNA molecules. However, heterozygous mutation carriers will generate 4 different types of DNA duplexes: 2 homoduplexes and 2 heteroduplexes. These 4 duplex molecules have each a specific temperature at which the DNA strands will separate. When loaded and run on a polyacrylamide gel in which an increasing denaturing gradient has been build up, fragments will migrate until they reach a position in the gel where the DNA strands will separate. Since a 40 base pair long sequence with high GC content (the GC clamp) was added artificially at one end of the PCR fragment (via a modified PCR primer), the DNA strands will not totally separate but remain attached at one extremity. This will result in the generation of a conformation that prevents further migration through the gel. Heterozygous mutant samples will thus show 4 bands after gel migration, control samples only one. The DGGE method works well on 200-300 base pair long DNA fragments, and is thus adequate for the analysis of small exons. In contrast to the PTT method, DGGE picks up the missense mutations, but since most of them are benign polymorphisms, many "false positives" will be identified.

The two techniques presented here above are able to detect mutations affecting only one or a few nucleotides (missense, nonsense, insertions or deletions a few nucleotides long). However, more recently several groups reported deletions or duplications involving one or several consecutive exons. These mutations are probably generated when a recombination occurs between mis-

aligned sequences. Since the intronic sequences of BRCA1 are very rich in repetitive elements (e.g. Alu repeat), misalignments and subsequent recombinations can be expected and are indeed observed. Such mutations are almost not encountered in the BRCA2 gene. In the past, mutations involving large stretches of DNA could be identified using time consuming techniques such as Southern blots, but recently a new approach was developed (MLPA) that is currently used for the routine screen of BRCA1.

We applied the BRCA genetic analysis on 186 Slovene families, and a cancer predisposing mutation was detected in 57 of them. Although the mutations were dispersed over the whole length of the BRCA1 and BRCA2 coding sequences, 5 distinct mutations occurred very frequently in the population. This clustering has very important consequences for the genetic screen. Indeed, since the analysis of only 4 PCR fragments led to the identification of the cancer predisposing mutations in 39 of the 57 BRCA1/2 positive Slovene families (68%), the genetic screen will always be initiated at these 4 particular DNA regions. Moreover, persons or families who do not strictly meet the including criteria for the genetic screen can be submitted to a restricted analysis (e.g. women with bilateral breast cancer or women who developed breast and ovarian cancer). A population based study can eventually be performed on the Slovene population to estimate the frequency of BRCA mutations in isolated breast cancer patients (male or female).

One of the mutations frequently occurring in the Slovene population (IVS16-2A>G) was found exclusively in Slovenia or at the Slovenian border in Italy, while 2 other recurrent mutations (1806C>T and 5382insC) have been encountered in many other countries. Very unexpected is the frequent occurrence of cancer predisposing missense mutations affecting one of the conserved residues that define the RING finger motif located near the protein NH_2 terminus (encoded by exon3 and exon5 of BRCA1).

In breast cancer only families (no cases of ovarian cancer) with 5 or more cancer patients we could identify a BRCA1/2 mutation in about 50% of the families. When less breast cancer cases occurred in the family, the mutation recovery rate also decreases (e.g. only 12% in families with 2 breast cancer cases). A possible explanation for this observation is the occurrence of several sporadic breast cancers within a same family. A similar decrease of the recovery rate is not observed however in families with breast and ovarian cancer. In those families the mutation recovery rate is 50% independently of the number of cancer affected relatives, confirming the hereditary nature of the disease even in families with a restricted number of cancer patients. Since the highest observed mutation recovery rate in Slovene families is 50% even in those families with a high number of affected relatives (and with ovarian cancer), we may suppose that a whole set of cancer predisposing mutations escape to our attention. At least part of them might be located in BRCA1 and/or BRCA2 but are not detectable with the currently used screening techniques (e.g. mutations located in the promoter region or within intronic

sequences distal from the intron/exon junctions). Mutations located in other genes can also be responsible for the frequent occurrence of breast (and ovarian) cancer in a fraction of the families. However such genes, with a clear dominant cancer predisposing phenotype comparable to what is observed with BRCA1 or BRCA2 mutations, have not been reported yet.

References

1. Ford D, Easton DF, Stratton M, Narod S, Goldgar D, Devilee P, Bishop DT, Weber B, Lenoir G, Chang-Claude J, Sobol H, Teare MD, Struewing J, Arason A, Scherneck S, Peto J, Rebbeck TR, Tonin P, Neuhausen S, Barkardottir R, Eyfjord J, Lynch H, Ponder BA, Gayther SA, Zelada-Hedman M and the Breast Cancer Linkage Consortium. Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. *Am J Hum Genet* 1998; 62:676-89.
2. Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, Liu
3. Q, Cochran C, Bennett LM, Ding W, et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* 1994;266:66-71.
4. Wooster R, Bignell G, Lancaster J, Swift S, Seal S, Mangion J, Collins N,
5. Gregory S, Gumbs C, Micklem G. Identification of the breast cancer susceptibility gene BRCA2. *Nature* 1995;378:789-92.
6. Krajc M, De Greve J, Goelen G, Teugels E. BRCA2 founder mutation in Slovenian breast cancer families. *Eur J Hum Genet* 2002; 10:879-82.
7. Teugels E, De Brakeleer S, Goelen G, Lissens W, Sermijn E, De Greve J. De novo Alu element insertions targeted to a sequence common to the BRCA1 and BRCA2 genes. *Hum Mutat* 2005; 26:284.