

EMQN Best Practice Guidelines for Molecular Genetic Analysis in Hereditary Breast/Ovarian Cancer

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These guidelines are in part based on the previous EMQN guidelines drafted by Clemens Müller-Reible and A. Haworth published in 2001.

Preamble

The first cancer genetic centres were opened in the late 1980's and early 1990's, at the time when our understanding of the genetic aspects of inherited cancer susceptibility was evolving fast. Following the discovery of the major genes in familiar breast and ovarian cancer, *BRCA1* and *BRCA2*, in 1994 and 1995 respectively, an increasing demand for genetic testing in to predict future risks arose from the health care community. Since then, cancer genetics has become a major part of the workload of clinical genetics centres.

Guidelines for genetic counselling and laboratory quality assessment in cancer genetics have been developed at local and national levels both in Europe and in USA. The European Molecular Genetics Quality Network (EMQN) drafted best practice guidelines on breast/ovarian cancer genetic testing as a first attempt towards European harmonisation in 1999. In 2007, OECD issued general recommendations for molecular genetic testing. The best practice guidelines presented here are the results of discussions held by European breast cancer geneticists at a workshop in Würzburg, Germany, October 24th-25th, 2007, aimed at updating the 1999 EMQN guidelines. This workshop was supported and co-organised by the EU-funded Network of Excellence EuroGenTest.

A note on mutation nomenclature: Unfortunately, guidelines for a systematic nomenclature of sequence alterations in the human genome have been issued only recently (www.hgvs.org; see below). To facilitate cross-referencing to the published literature, we decided to use the BIC nomenclature in this document although we support the initiative for the use of a more systematic approach.

Introduction

Breast cancer remains the most common form of cancer in European women with a lifetime risk exceeding 10%. It exhibits familial aggregation in 5-10% of all breast cancer cases, where the familial clustering is compatible with the segregation of a dominant autosomal allele.

Germ line mutations in the two major susceptibility genes, *BRCA1* and *BRCA2*, confer high risks of both breast and ovarian cancer. Mutations in other genes like *TP53* and *PTEN* are rare, but also cause high breast cancer risks; whereas mutations in *ATM*, *CHEK2*, *PALB2* and other less well-characterized repair/checkpoint genes attribute a lower (two-fold) risk of breast cancer (Walsh & King 2007). Taken together, the known susceptibility genes account for less than one third of breast cancer families undergoing genetic testing while other gene defects remain to be discovered.

However, no further high-risk breast cancer genes have been identified to date and while this may be due to genetic heterogeneity and the presence of multiple rare or population-specific susceptibility alleles (Erkko et al., 2007). This could be due to the contribution of a combined influence of alleles that individually only confer a moderate or low risk. Association studies have provided evidence of such common low-risk variants in *CASP8*, *FGFR2*, *TNCR9*, *MAP3K1*, *LSP1* as well as for SNP markers on chromosome 2 (2q35) and chromosome 16 (16q12), (Cox et al., 2007, Easton et al., 2007, Stacey et al., 2007). From a clinical point of view, while breast cancer susceptibility may be largely polygenic (Pharoah et al., 2002), *BRCA1* and *BRCA2* still remain the only genes of significance for patients with a personal or family history of breast/ovarian cancer. The multi-allelic character of both genes, with more than 3200 described sequence variants (Breast Cancer Information Core (BIC) Database:http://www.ncbi.nlm.nih.gov/Intramural_research/Lab_transfer/Bic/), poses a significant challenge with respect to accurate detection, characterization and interpretation of sequence variants for use in counselling, screening and disease prevention.

***BRCA1* and *BRCA2* genes**

Evidence for the existence of *BRCA1* was first provided by linkage analysis of a single locus for early onset breast cancer to chromosome band 17q21 in 1990 (Hall et al., 1990) and the gene was identified four years later (Miki et al., 1994). *BRCA2* was mapped to chromosome 13q13 soon after (Wooster et al., 1994) and cloned a year later (Wooster et al., 1995; Tavtigian et al., 1996). *BRCA1* spans over 81 kbp genomic

DNA and contains 22 coding exons encoding an 1863 amino acid nuclear phosphoprotein. *BRCA2* extends over 84 kbp, contains 26 exons and encodes a 3418 amino acid protein.

BRCA1 and *BRCA2* encode proteins that are mainly involved in DNA repair. In addition, the *BRCA1* protein is involved in transcriptional regulation, ubiquitinylation and in cell cycle checkpoint control for G2 and S-phase arrest (Venkitaraman et al., 2004). Since both *BRCA* genes are important for genome maintenance, loss of function of these genes induces genomic instability. However, the exact mechanism that leads to breast and ovarian cancer development is still unknown. It seems likely that cancer development occurs from different mammary cell types and/or along specific progression pathways as evidenced by the distinct tumour phenotypes: Tumours in *BRCA1* mutation carriers are largely oestrogen-receptor (ER)- and HER2-negative (basal-like subtype), whereas tumours in *BRCA2* mutation carriers often display an ER-positive phenotype (luminal subtype) (Sørlie et al., 2003). Although somatic mutations in *BRCA1* and *BRCA2* are rare, both genes can be affected by a large variety of germline alterations, leading to an inherited cancer predisposition.

***BRCA1* and *BRCA2* mutations**

Today, thousands of different sequence variants of *BRCA1* or *BRCA2* have been identified through the analysis of breast cancer families and population-based case studies (Breast Cancer Information Core, BIC). These can be broadly classified into mutations that are known or likely to be deleterious and disease-associated; variants of unknown function (unclassified variants, UVs); and genetic variants that are likely to be neutral and without clinical importance. Deleterious mutations can have different effects on the protein product: (1) Premature translation termination codons leading to protein truncation and/or degradation/instability of truncated proteins or loss of expression due to nonsense-mediated mRNA decay (NMD), (2) Single amino acid substitutions leading to dysfunctional full-length translation products, or (3) Regulatory lesions resulting in loss of expression or effects on mRNA splicing.

Protein truncating mutations result from nucleotide insertions or deletions that introduce shifts in the reading frame leading to a premature stop codon (frameshift

mutations), or nucleotide substitutions that generate an immediate stop codon (nonsense mutations). Alterations in the consensus splice acceptor or donor sequence sites of exon-intron junctions are also usually deleterious. Splice consensus sites are defined as the intronic nucleotides (usually 3 to 6) immediately flanking an exon. Alterations at the first and second position from the exon (+/- 1 or +/- 2) often lead to abnormally spliced mRNA messages, either by exon skipping or by use of cryptic splice sites. Sequence variants at the more distal positions (+/- 3 to +/- 6) and at the first/last nucleotide of an exon may also alter mRNA splicing, although in a less predictable manner. Collectively, frameshift, nonsense and splice site mutations are predicted to result in reduced transcript or protein level due to NMD and/or degradation/instability of truncated proteins or to give rise to truncated proteins with completely or partially reduced function. All *BRCA1* and *BRCA2* truncating mutations are considered to be deleterious, with the exception of alterations within the extreme carboxy terminal region of *BRCA2*, downstream of the polymorphic stop codon K3326X. Risk interpretation for premature stop codon mutations falling within exon 27 of *BRCA2* should therefore be made with caution.

Single amino acid substitutions can be classified as deleterious mutations when they affect the highly conserved residues in the *BRCA1* RING domain, e.g. Cys44 and Cys61. Substitutions involving these amino acids have been shown to disrupt heterodimerization of *BRCA1* with BARD1 and/or E2 ubiquitin conjugating enzymes, resulting in loss of the E3 ubiquitin ligase activity. This subsequently affects *BRCA1* tumour suppressor activity, either via an effect on DNA damage repair, centrosome duplication, transcription or chromosome decatenation (Morris et al., 2006). The *BRCA1* carboxy-terminal BRCT domains (shown to bind phosphopeptides and interact with numerous proteins involved in cell cycle regulation and DNA repair) are also known to harbour deleterious missense mutations. Evidence from several approaches such as functional (transactivation) assays, sequence homology, chemical differences (Grantham scores), inferred embryonic lethality, and linkage/segregation analysis supports the deleterious effect of a number of *BRCA1* BRCT missense variants. Confirmed deleterious *BRCA2* missense mutations are still rare but may occur in the DNA-binding domain (comprising amino acids 2500-3098) (Easton et al., 2007). In summary, the majority of missense variants (and in-frame insertion/deletions of single or multiple amino acids) in *BRCA1* and *BRCA2* must be

referred to as unclassified variants. For other amino acid substitutions (some of them very common) an effect on the protein function has been ruled out and these are therefore referred to as neutral sequence variants of no clinical importance. This may also be the case for the majority of synonymous variants, i.e. nucleotide substitutions that do not lead to an amino acid change. However, these silent variants must also be interpreted with caution (especially when they appear to be novel and appear to be associated with disease in a family), since they may create cryptic splice sites, disrupt splicing enhancers (ESE) or silencers (ESS) in exons, or alter the translation rate (tRNA availability). The occurrence of predisposing mutations residing in the 5' or 3' untranslated regions (UTRs) of the transcripts is still unknown but should not be excluded. Guidelines by Bell et al set standards for the interpretation and reporting of sequence variants of unknown pathogenicity in genes involved in disorders with Mendelian inheritance (Bell et al., 2007)

Germ line mutations may also comprise complex genomic rearrangements, including deletions and duplications of large regions that escape detection by non-quantitative PCR-based mutation screening of genomic DNA templates, due to removal of primer sites. Genomic rearrangements typically arise from unequal homologous recombination between short interspersed nuclear elements such as *Alu* repeats, which are present at high density in the *BRCA1* gene. More than 40% of the 81 kb *BRCA1* region consists of *Alu* elements. These large genomic alterations usually involve multiple exons and lead to nonsense transcripts. They may also result in in-frame transcripts with loss or gain of sequence, in which functionally important regions may be retained. In these latter cases, interpretation of the subsequent effect is more difficult. Some germ line chromosomal *BRCA* rearrangements may have breakpoints residing 5' and/or 3' of the genes, affecting regulatory regions and occasionally adjacent genes or pseudogenes such as *psiBRCA1*. In addition, the presence of regulatory mutations solely affecting non-coding sequences and located either adjacent to the genes or at deep intronic sites, is anticipated but not documented as yet.

Founder mutations

The past decade has witnessed the comprehensive characterization of *BRCA1* and *BRCA2* mutations in different populations. Founder mutations have been identified in many countries such as Iceland, Norway, Poland, the Baltic countries, the Netherlands and Belgium; while in countries with ethnically mixed populations the range of genetic variation is wide (Peelen et al., 1997). Three mutations, two in *BRCA1* (185delAG and 5382insC) and one in *BRCA2* (6174delT) are commonly found in the Ashkenazi Jewish population (Struewing et al., 1997). If none of the commonly found mutations is detected in a family with Ashkenazi ancestry, the laboratory should consider a full *BRCA1/2* screen. Similarly, the prevalence of a single mutation (*BRCA2* 999del5) in Iceland may impact on screening strategy decisions for that population (Tulinius et al., 2002).

Penetrance

The risk of developing breast and/or ovarian cancer is not identical for all carriers of *BRCA1* and *BRCA2* mutations. The complexity of risk prediction is due to the considerable effect of environmental, other genetic and hormonal factors as modifiers of the gene defect (Narod, 2006; Andrieu et al., 2006; Chang-Claude et al., 2007; Brothet et al., 2007, Antoniou et al., 2007). For instance, a SNP in the 5' UTR of RAD51, 135G>C, that affects splicing has been shown to significantly modify breast cancer risk in *BRCA2* mutation carriers, with a odds ratio of 1.17 for heterozygotes and 3.18 (95% CI 1.39-7.27) for the rare homozygotes (Antoniou et al., 2007). Nevertheless, female *BRCA1* and *BRCA2* mutation carriers have a substantially higher risk for breast and ovarian cancer compared to the population in general. In families studied by the Breast Cancer Linkage Consortium (BCLC), *BRCA1* mutation carriers had by the age of 70 years a breast cancer risk of 71% (95% CI 53-82%) and a risk for ovarian cancer of 47-63% (Easton et al., 1995). Carriers of *BRCA2* mutations had by age of 70 years a breast cancer risk of 84% (95% CI 43-95%), and a slightly lower risk of ovarian cancer as compared to *BRCA1* carriers, 27% (95% CI, 0-47%) (Ford et al., 1998). However, these risks were lower in a meta-analysis of families with a *BRCA1/2* mutation identified through population-based studies of breast and ovarian cancer, where *BRCA1* mutations were estimated to confer a cumulative risk of breast

cancer of 65% by age of 70 (Antoniou et al., 2003). The corresponding risk for *BRCA2* mutations was estimated to be 45%. Furthermore, truncating mutations located in the ~3kb central portion of the *BRCA2* gene, the ‘ovarian cancer cluster region’, have been associated with an increased risk of ovarian cancer and slightly decreased risk of breast cancer (Thompson et al., 2001).

Context for genetic testing

Molecular genetic testing should only be performed in the context of genetic counselling. *BRCA1* and *BRCA2* analysis is preferably carried out on a blood (or other normal tissue) sample from an affected individual, if possible an early onset case. If this analysis results in no mutation despite a high prior probability and sensitive mutation screening technique, one should consider testing a second case from the family to exclude the possibility of a sporadic case (‘phenocopy’) being tested in the first instance. If a disease-associated mutation is found, presymptomatic testing of other family members for the specific mutation is possible. A written informed consent should be obtained from all patients prior to storage or analysis of their sample.

Techniques for mutation detection

The choice of mutation detection technique used largely depends upon local preferences and facilities, and it is not possible to recommend a single established technique. One laboratory may become experienced and skilled in using a certain assay that performs sub-optimally in another laboratory. The source of material for testing varies as well, but most laboratories extract genomic DNA from blood samples. It is recommended that RNA is extracted and stored for future analysis or to confirm splice site mutations. The use of RNA/cDNA as a template for mutation screening as a first-line strategy is not recommended however, as variation in quality of RNA/cDNA can cause problems. In addition, there may be difficulties in interpreting data generated following RNA/cDNA mutation screening due to the presence of spurious fragments caused by alternative splicing (false positives), or due to NMD (false negatives). Using genomic DNA as a template, a screening strategy usually involves the analysis of each coding exon, together with their flanking

intronic sequences. Larger exons such as *BRCA1* exon 11 and *BRCA2* exons 10-11 are divided into multiple overlapping fragments. Care must be taken when designing PCR primers to avoid sequence variants (e.g. SNPs) in primer binding sites that could result in allele-biased amplification.

A number of scanning methods are available for detection of sequence alterations. These pre-screening methods do not identify the specific underlying sequence alteration however, when used as part of the mutation screening strategy, a pre-screening method significantly reduces the overall sequencing workload. These methods are usually based on the difference in structure, melting and/or migration property of the mutant and wildtype fragments, respectively.

Direct sequencing

Direct sequencing of PCR fragments using chain-terminating dideoxynucleotide chemistry according to Sanger (dye terminator sequencing), is often quoted as the gold standard in mutation analysis since it provides direct information of the actual order of nucleotides. In reality, the sensitivity is often not as high as anticipated, due difficulties in standardizing DNA quantity, integrity and purity and difficulties in balancing the reaction mixture and clean-up to avoid dye-blobs and spikes; high background or low peak signal intensity; or in reading through regions with GC-rich, compressed, secondary structure or homopolymeric sequences. However, for *BRCA1* and *BRCA2* a well established protocol is available, which comprises a carefully designed set of PCR primers with uniform PCR-conditions, tagged with universal sequences thus making standardised analysis in 96-well format possible (National Genetics Reference Laboratory, Wessex; <http://www.ngrl.co.uk/Wessex/downloads>.) Available sequence analysis software packages allow for a quick and efficient data analysis. With careful design and standardization, dye terminator sequencing remains a gold standard to which other techniques should be compared. Recently developed next-generation non-Sanger sequencing technologies, based on massive parallel sequencing of individual DNA fragments, show great promise in setting new standards in speed and cost of sequence analysis.

Denaturing High Performance Liquid Chromatography (DHPLC)

DHPLC relies upon differential denaturing profiles of heteroduplex and homoduplex DNA molecules (PCR fragments). These are separated by ion-pair, reverse-phase liquid chromatography on a special column matrix using partial heat denaturation and an increasing concentration of acetonitrile. A temperature profile specific for each PCR fragment must be applied in order to obtain a maximal temperature difference during melting analysis. The technique requires extensive optimisation of conditions for each exon/PCR fragment, and multiple temperature running conditions may be required to reach acceptable sensitivity over the entire length of a fragment. Although the analyses are carried out sequentially, throughput is very high and can be fully automated (96-well format). Special or labelled primers are not required and the running cost per sample is low, compensating for the high initial investment for the instrument. Sensitivity is high, well above 90% for most fragments. All types of sequence variants are detected except for large genomic rearrangements. Although frequent polymorphisms may be recognised by their characteristic melting/elution profile, all aberrant fragments must be subsequently characterized by direct sequencing.

Hi-Res Melting technology

Using the same principle of differential melting profiles of homo- and heteroduplexes, the Hi-Res Melting (HRM) technique enables the detection of sequence variants by monitoring fluorescence changes of an intercalating double-stranded DNA binding dye induced following denaturation using an increasing temperature profile. Benefits of HRM technology include rapid and fully automated analysis from 96- or 384-well plates, the absence of the need for special primers, and convenient programs for the detection of variants from high-resolution data both on the temperature axis and fluorescence axis. The sensitivity of the technique compares well to DHPLC.

Other methods using differential mobility of fragments

Conformation-sensitive gel electrophoresis (CSGE), heteroduplex analysis (HA), denaturing gradient gel electrophoresis (DGGE), as well as the methods developed from these techniques, e.g. fluorescence-based CSGE (F-CSGE), have all been initially optimized for use with polyacrylamide gels and subsequently modified for use with capillary-based instruments. When optimized, these methods offer a good or

even excellent sensitivity; they have the capacity to detect all types of small mutations, and are a low-cost alternative to DHPLC for mutation scanning. However, these methods are gradually being replaced by other more effective and less laborious scanning techniques.

Protein Truncation Test (PTT)

Unlike the previously mentioned techniques, this method aims at detecting only mutations that result in premature termination of protein synthesis. When applied using genomic DNA as template, PTT is most suitable for screening the large exons in *BRCA1* and *BRCA2*, since a fragment of at least 1000bp is required for optimal resolution. The method comprises a combined *in vitro* transcription-translation assay, it is relatively easy to perform and requires no expensive equipment. The large exons are divided into several fragments with an overlap that is large enough to detect mutations that may be positioned close to the ends of the fragment. The 5' and 3' ends of each large exon must be scanned by other methods or directly sequenced, in order to detect putative splice site mutations. In view of the numerous unclassified variants in exon 11 of both BRCA genes, the advantage of PTT is that it only detects deleterious mutations. Disadvantages of PTT include the fact that it is a multi-step procedure and therefore may have an associated increased potential sample mix-up risk. In addition, PTT does not detect putative missense mutations and the technique requires the use of a radioisotope (^{35}S -methionine).

Screening for large rearrangements

Techniques to detect complex genomic rearrangements include RNA/cDNA-based assays such as PTT and DNA-based assays such as Southern blotting, colour bar coding, long-range PCR, quantitative multiplex PCR of short fluorescent fragments (QMPSF), semiquantitative multiplex PCR, DNA microarrays, and multiplex ligation-dependent probe amplification (MLPA). MLPA has rapidly become popular due to its ease of use, economy with respect to laboratory time and the low amount of sample required. MLPA has certain drawbacks however, including false positive scoring of 'single-exon deletions' due to sequence variants in probe binding sites or false negative scores due to the restricted coverage of the exon-specific probes. In addition, MLPA provides only limited clues to the location of deletion or duplication breakpoints in the usually very large intronic or flanking regions, resulting in the need

for laborious mapping for sequence characterization of the rearrangements. MLPA constitutes a convenient addition to the analytical tools for *BRCA* gene screening, but it should be emphasized that any positive results from an MLPA run should be confirmed by an independent assay. For *BRCA1* a second kit is available. For *BRCA2*, sequence confirmation of the breakpoints or another method allowing detection of large rearrangements (RT-PCR, Southern blot, QMPSF, a mutation specific assay etc) should be applied.

Mutation-specific tests

A multitude of techniques are available to test for a specific mutation at a given position in the gene. These include assays such as restriction enzyme digestion, real-time PCR using TaqMan probes, allele-specific primer extension using the amplification refractory method (ARMS), allele-specific single-base extension (e.g. Illumina Infinium II assay), allele-specific hybridization (e.g., with chip-based technology) or ligase chain reaction (e.g. Illumina GoldenGate assay). Sequenom has developed a matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry approach to detect SNPs by the differences in mass of modified base terminators incorporated into a single base extension, lending itself to multiplexing by variable primer length. The pyrosequencing technology utilizes the release of a pyrophosphate (PPi) for each dNTP that is incorporated into the new DNA strand. A sulfurylase converts PPi to ATP in the presence of APS (adenosine phosphosulfate), and ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates detectable light that is proportional to the number of nucleotides incorporated. Addition of dNTPs is performed one by one, each time followed by addition of apyrase, that continuously degrades unincorporated dNTPs and excess ATP, and a pyrogram is gradually built up. An advantage of this technique is the ability to read a sequence immediately following the primer, which makes sequencing of short fragments (i.e. from formalin-fixed paraffin-embedded tissue, FFPE) possible. Disadvantages, using the standard set up, are the relative short reads and difficulties in analysing homopolymeric sequence. These and related techniques may be of considerable value when limiting the genetic screening to previously known or founder mutations, especially in large cohorts.

Linkage and segregation analysis

Linkage analysis of extensive breast cancer families using microsatellite or SNP markers was used to map the location of both *BRCA1* and *BRCA2* (Hall 1990, Wooster 1995). Although linkage analysis only provides information on the chromosomal position of the disease-causing alteration, the power of this approach has been demonstrated for instance, by repeated analysis of linked families and the subsequent unmasking of previously hidden mutations, such as large rearrangements. Linkage analysis is not suitable as part of diagnostic practice and is not offered as routine service, but segregation analysis may be useful for the evaluation of unknown variants and for the interpretation of their association with disease.

Controls

In order to monitor sensitivity and specificity during routine mutation screening, positive controls should be used at an appropriate frequency. For predictive testing, a sample from a close relative carrying the familial mutation is preferred. Only if a sample from a relative is not available should a sample from an unrelated individual containing the relevant mutation be used. It is advisable to confirm the presence of a particular mutation within a family, using an independent sample, prior to offering predictive testing. The regular use of positive controls is essential for all mutation-specific tests, e.g. if a laboratory tests for founder mutations only.

Validation of analytic procedures and internal quality control

No recognised ‘reference methods’ have been agreed upon for molecular genetic analysis. Although direct sequencing is frequently referred to as the ‘gold standard’, experience from external quality assessment schemes shows that the error rate may be as high as 3-5 % (Mueller et al. 2004; Patton et al. 2006). The performance characteristics of pre-screening methods have often been established on small series of samples and their sensitivity and robustness may be over-estimated. Therefore, diagnostic laboratories are obliged to fully validate any analytical procedure, which they introduce into their genetic service. The validation should be performed before a test is approved for routine application. Validation comprises as a minimum, the determination of an estimate of test specificity, sensitivity and robustness. These parameters are established by testing a series of samples of known genotype (control materials). Such materials may be obtained from other laboratories, or may comprise

samples that have been tested previously by an independent method. For the purpose of validation of screening methods samples containing published SNPs can be equally useful. Given the size of the BRCA genes, it is not realistic to have access to control materials for each PCR fragment. However, labs should establish a collection of control materials and collaboration with other laboratories may be helpful in achieving this. Requirements for validation may vary in detail with the methodology used.

Quality Assurance

Any laboratory offering genetic testing within a health care system has to consider national and international regulations for the tests they deliver so that patient safety and privacy will not be compromised. Written Standard Operating Procedures for the methods used is mandatory. Laboratories should have procedures in place, which allow for an internal audit and control of the whole analytical process, from sample receipt to report writing. The implementation of a laboratory quality management system, e.g. through accreditation according to ISO 15189 or 17015 is recommended (see OECD guidelines for details). To maintain competence, laboratories should regularly participate in external quality assessment schemes available (EMQN, UKNEQAS,CAP).

Screening strategies

Given current knowledge and for mixed European populations, molecular analysis for heritable breast/ovarian cancer cannot be regarded as complying with best practise if the BRCA1/2 genes are only analysed for the most common mutations. Exceptions may include populations with known prevalent founder mutations (e.g. Iceland, Norway) and patients of Ashkenazi Jewish ancestry where the three founder mutations account for over 90% of the familial breast/ovarian cancers. Laboratories, which decide to test for founder mutations only, should make arrangements with another laboratory to ensure that a fully comprehensive screen is available to the patient.

Genomic deletions or duplications account for 5-10% of all germ line mutations in *BRCA1* and for a lesser fraction in *BRCA2*. It is cost-effective to screen for large

genomic rearrangements as the first step of the diagnostic algorithm (Walsh et al., 2006).

With all ‘whole-gene’ approaches, it is considered best practise to analyse both genes, *BRCA1* and *BRCA2* to completion, even if a disease associated mutation has been detected in the early steps of the analysis.

***BRCA* gene mutation nomenclature**

Traditionally, the description of *BRCA1* and *BRCA2* mutations has followed the nomenclature used by the BIC consortium and database (containing several thousands of different sequence variants) and this has been applied to a vast number of publications during the past 12 years. For numbering of nucleotides, BIC makes reference to the *BRCA1* sequence GenBank No. U14680 and *BRCA2* sequence GenBank No. NM_000059 both starting somewhere in the non-coding 5'UTR upstream of the first ATG. Unfortunately, the description of sequence variants is not uniform among scientific journals or among oncogenetic centres and this is a serious source of confusion and misinterpretation. In an attempt at harmonisation, the Human Genome Variation Society (HGV; <http://www.hgvs.org/>; den Dunnen et al., 2000) has emphasized the need for the use of a more standardised, systematic nomenclature and has issued detailed guidelines to this effect; while at the same time, being aware of the advantages and disadvantages of changing an accepted formula. One may argue that introducing a change in well established mutation descriptions will cause more harm than good, therefore, both ways of describing mutations, HGVS and BIC, should always be used in parallel during a transitional period. Moreover, in some disciplines the term ‘mutation’ is used to indicate ‘a change’ while in other disciplines it is used to indicate ‘a disease-causing change’. Similarly, the term ‘polymorphism’ is used both to indicate ‘a non disease-causing change’ or ‘a change found at a frequency of 1% or higher in the population’. To prevent this confusion, HGV recommends that neutral terms like ‘sequence variant’, ‘alteration’ and ‘allelic variant’ should be used in place of ‘mutation’ and ‘polymorphism’ (including Single Nucleotide Polymorphism)

Reporting

General information on requirements for reporting can be found in the OECD Guidelines for Quality Assurance in Molecular Genetic Testing (www.OECD.org), and in the guidelines issued by the Swiss Medical Genetics Society (http://sgmg.ch/user_files/images/SGMG_Reportin...).

For the interpretation of an observed sequence variant it is essential to establish the causal role of the variation in the pathogenesis of the disease. Many clinical geneticists have specialized in cancer genetics and thus should be aware of the full mutation spectrum of each gene and be familiar with the techniques used in molecular genetic analysis. Reports should include a statement of which genes were tested and why, the extent and the limits of the analysis and the methods used.

Disease-associated mutation

The following mutation types are likely to have pathological consequences for the protein function:

- Mutations that are predicted to formally interfere with protein synthesis
- Mutations that are likely to lead to altered splicing of the mRNA (splice site mutations). It is strongly advocated that nucleotide substitutions other than splice site mutations affecting the -1, -2 or +1, +2 intronic nucleotide positions are referred for *in vitro* functional studies performed in research laboratories to determine whether or not they cause aberrant splicing leading to transcripts with a premature stop codon.
- Other mutations with experimental evidence (published or own data) of their impairment of the protein's function (the Breast Cancer Core)
- Sequence variants that have been shown to strictly co-segregate with the disease in several unrelated families.

All other sequence variants must be considered as “unclassified” until functional evidence becomes available.

Currently, the type/site of mutation in the *BRCA1* or *BRCA2* gene does not affect clinical management. There is evidence that some mutations may confer a higher risk of ovarian cancer than others, and further research may provide evidence that certain

mutations are associated with a lower penetrance. However, due to the early stage of this work, it is not considered appropriate to modify the advice given with respect to clinical management in relation to the type/site of the mutation identified.

Finding a causative mutation in an affected index case

The report should state that a disease associated mutation could be detected and that there is now a basis for predictive testing of other family members at risk of developing early breast/ovarian cancer, in addition to their own increased breast cancer risk (*BRCA2* carriers). In a male mutation carrier the report should indicate that the test result has implications for his relatives,

Not finding a mutation in an affected index case

As mentioned above, it is necessary to include details of the extent and limits of the analysis. If the analytical techniques do not cover certain mutation types (e.g. large genomic rearrangements) suggestions for additional tests should be made. In the case of a strong family history and a negative test result for *BRCA1* and *BRCA2* mutations, another affected family member should be tested, as the first patient could be a phenocopy. If the final result is still negative the report should state that a heritable predisposition to breast cancer cannot be excluded.

Pre-symptomatic testing

In comparison to the testing of affected index patients, predictive testing of at risk relatives for a known mutation requires additional precautions. Pre- and post-test genetic counselling is mandatory (see below) since important clinical and life planning decisions of healthy people may follow from the genetic test result. Procedures should be in place to ensure the integrity of sample handling and processing. For this reason, many laboratories request and test two independent samples to minimize handling errors.

Not finding the mutation segregating in the family

Some caution should be exercised in giving a population risk estimate to individuals found not to have the *BRCA1/2* mutation segregating in the family following presymptomatic testing. There is some evidence that their risk may still be a little above the population risk, possibly because of other shared susceptibility genes.

Finding the mutation segregating in the family

The report should state that this results in a high lifetime risk of breast/ovarian cancer.

Prenatal or preimplantation diagnosis

While prenatal or preimplantation diagnosis with the purpose of terminating an unwanted pregnancy or not selecting embryos carrying the mutation is offered as an intrinsic part of a clinical genetics service. This is rarely requested in the case of BRCA1/2. Requests for prenatal or preimplantation diagnosis should always be referred to a clinical genetics service.

Reporting unclassified variants and polymorphisms.

If the genetic testing procedure has identified an unclassified variant (UV) as the only sequence change, this should be reported as such. However, the report should clearly state that the clinical significance of the variant is unknown, its identification does not provide a basis for changing the clinical management of the patient or for offering predictive testing to at risk relatives.

Practices vary between countries and laboratories with regard to whether a polymorphism should be reported. However, where polymorphisms are reported, the text should be completely clear that the variant has been confirmed as being not clinically relevant. This is particularly important when a polymorphism is identified in the same case as a disease-associated mutations or an unclassified variant, or when identified as the only sequence change.

BIC database

Reporting of novel variants to BIC (www.nhgri.nih.gov/intramural_research/lab_transfer/bic/) is important for the collection of mutation data and is encouraged. It is essential to include the evidence for clinical relevance with the submission of every variant.

Testing for other breast cancer susceptibility variants

Despite intensive research, no single gene has been shown to be sufficiently prevalent and penetrant as a candidate for a third breast cancer gene. However, a number of

genes have been studied in view of their potential contribution to cancer predisposition and/or modification of penetrance of a *BRCA1/2* mutation, e.g. the RAD51, 135G>C variant (Antoniou et al. 2007). In general, the odds ratio for breast/ovarian cancer risk is only slightly modified by each individual variant (Walsh and King, 2007). It can be envisaged that multi-parametric risk estimates based on the combined analysis of several such variants may generate odds ratios that are sufficiently high to have clinical implications, e.g. to suggest the starting of mammographic screening at a younger age than that recommended for the general population. There is also some evidence emerging that women with certain genotypes may be more likely to have cancers of a particular histological type and this could have implications for screening or treatment. However, before this type of advice can be implemented in clinical practice, it needs to be based on reliable data; therefore more extensive research studies in large cohorts of women are required. The authors of these guidelines consider that at present this work is at too early a stage for the testing of such variants or SNPs to be used clinically.

Future of genetic testing

During the past decade genetic testing for *BRCA1* and *BRCA2* has taken an important place in medical practice. In future, further knowledge of specific molecular signatures of *BRCA1* and *BRCA2*-associated cancers will promote the development of new targeted therapies. Currently, trials with cisplatin and PARP inhibitors for treatment of breast and ovarian cancer in women carrying mutations in *BRCA1* and *BRCA2* are being run by Breakthrough Breast Cancer in UK by Tutt and Ashworth. Furthermore, the increasing knowledge of other susceptibility genes will pose a challenge to clinical practice.

Clinical validation

Although the focus of this document is the quality of the laboratory test, the clinical validation for the molecular test offered should also be of concern to the laboratory geneticist. The clinical validation for breast cancer genetic testing includes pre-selection of individuals and families offered genetic testing, genetic counselling and clinical surveillance for those with a substantially increased risk. In many European countries, there are national best practice programmes for clinical validation, and these may vary. For those countries with no national standards; the UK, French, or

German health service-provided professional programmes might be a helpful reference to laboratories looking to implement recommended standards (<http://www.nice.org.uk>, Eisinger et al., 2004, Kuschel et al., 2006).

Pre-selection of patients being tested

Given the limited resources allocated to health care by the different national governments in Europe, a population-wide screening is not available, nor is it possible to offer a mutation analysis on demand. Usually, pre-selection of patients is done on the basis of a risk calculation based on the previous family history. This family history should at least contain information on occurrence of cancers among first, second and third degree relatives. The information needed includes the type of cancer, age at diagnosis and if possible, the results of a pathological examination. Based on this information, several algorithms have been developed and are used for clinical risk estimation, and as entrance criteria to genetic testing and clinical follow up programmes.

The typical pattern for a family where a *BRCA1* mutation is segregating is the occurrence of multiple cases of breast and ovarian cancer at a young age revealing a dominant pedigree pattern of inheritance. For *BRCA1*-related breast cancer, median age at diagnosis is 35-40y and for ovarian cancer, 45-50y. Other cancers are rarely seen, perhaps with the exception of prostate cancer which has been slightly overrepresented in some studies. For families where a *BRCA2* mutation is segregating, male breast cancer is not infrequent. A *BRCA2* mutation occurs in 10 percent of male patients with breast cancers and no family history of breast and ovarian cancer.

Typically, when a dominant pedigree pattern emerges, an index patient who has or had breast or ovarian cancer will be offered testing for *BRCA1* and *BRCA2*. The mutation detection rate in a given set of breast or breast/ovarian cancer families is dependent upon family selection. *BRCA1* mutations and to lesser extent, *BRCA2* mutations are most frequently identified in multi-case breast/ovarian cancer or ovarian cancer families, while a *BRCA2* mutation is more likely to be found in families including male breast cancer. Age at onset and bilateral status, as well as

tumour phenotype, are also criteria that can be taken into consideration when estimating the likelihood of detecting a deleterious *BRCA* gene mutation. On the basis of individual patient counselling, it is useful to assess the prior risk by Claus risk calculations or BRCAPRO (Berry et al, 2002). As an alternative, BOADICEA (Breast and Ovarian Analysis of Disease Incidence and Carrier Estimation Algorithm; Antoniou et al., 2004) uses detailed family history to predict a woman's risk of developing cancer, also taking into account other genes besides those of the well-known *BRCA1* and *BRCA2* genes (www.srl.cam.ac.uk/genepi/boadicea).

Genetic counselling

Genetic counselling is an integrated part of genetic testing. It should ideally be performed by a clinical geneticist or an oncologist trained in genetic counselling. There are established protocols for pre-test counselling, particularly for predictive testing reference. The first session is usually a first information-exchanging session which includes a discussion of the implications of carrying the mutation detected in the family (once it has been characterised); the risk to the individual of inheriting the mutation and the risk-reduction options available if the consultand is found to be mutation-positive. There should also be discussions about the implications of the test for other family members. This session is normally followed in some weeks or months by an appointment at which blood is drawn for the test. Written informed consent should be obtained for all genetic tests. The results should usually be given by personal communication to the consultand as soon as available and appropriate management organised for mutation carriers.

Surveillance programmes

The most important personal benefit of genetic testing for an already affected woman (usually the index patient) is her enrolment in a surveillance programme for the early detection of secondary tumours. Therefore, laboratories that offer genetic testing for the *BRCA1/2* genes should establish whether such programmes are available for their families. *BRCA1/2* mutation carriers should be offered annual breast cancer screening from 25 years of age. Ovarian cancer screening by trans-vaginal ultrasound and serum marker estimates are still of unproven benefit. Prophylactic surgical options such as salpingo-oophorectomy and mastectomy have been shown to substantially decrease future cancer risk and can be discussed as and when appropriate for the consultand.

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Appendix

Nomenclature

HGV recommends that all variants should be described at the most basic level, i.e. the DNA level, always in relation to a reference sequence, either a genomic or a coding DNA sequence. Although theoretically a genomic reference sequence seems best, in practice a coding DNA reference sequence is preferred and indicated by a letter 'c.' for coding DNA (cp. to 'g.' for genomic, 'r.' for RNA and 'p.' for protein sequence) DNA. Nucleotide 1 should be the A of the ATG-translation initiation codon, and UTR or intronic variants are described by a '−' or '+' N from the closest coding nucleotide. Substitutions are indicated by '>', while an underscore '_' indicates the range of a

deletion ('del'), duplication ('dup') or insertion ('ins'). In sequence repeat regions the most 3' position possible is arbitrarily assigned to have been changed. For example, *BRCA1* 185delAG (Stop 39), according to BIC style, will with HGV nomenclature read: *BRCA1* c.68_69delAG, GenBank U14680 (p.Glu23ValfsX16).