DESCRIPTION AND INTERPRETATION OF VARIOUS SNPs IDENTIFIED BY *BRCA2* GENE SEQUENCING

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Abstract: Molecular diagnosis for hereditary breast and ovarian cancer (HBOC) involves systematic DNA sequencing of predisposition genes like *BRCA1* or *BRCA2*. Deleterious mutations within such genes are responsible for developing the disease, but other sequence variants can also be identified. Common Single Nucleotide Polymorphisms (SNPs) are usually present in human genome, defining alleles whose frequencies widely vary in different populations. Either intragenic or intronic, silent or generating aminoacid substitutions, SNPs cannot be afforded themselves a predisposition status. However, prevalent SNPs can be used to define gene haplotypes, with also various frequencies. Since some mutation can easily be assigned to haplotypes (such is the case for *BRCA1* gene), SNPs can therefore provide usual information in interpreting gene mutations effects on hereditary predisposition to cancer. Here we describe 10 *BRCA2* SNPs identified by complete gene sequencing.

INTRODUCTION

Hereditary breast and ovarian cancer (HBOC) is mainly attributable to predisposition genes, whose mutant alleles with high or medium penetrance present variable risk magnitude [Ferlay et al., 2010]. Although at least 20 genes have already been associated with these types of cancer, their contribution in breast/ovarian cancer in different populations remain unknown [McClellan et al., 2010; Walsh et al., 2007]. New genes responsible for breast/ovarian cancer are expected to be identified as many families with hereditary breast/ovarian cancer are negative for mutations in all above genes. The model that is more favoured for breast cancer is not the “common disease – common alleles” but rather the “common disease – rare alleles”. To date, the major predisposition genes to HBOC are considered to be *BRCA1* and *BRCA2* [Narod et al., 2004], as lifetime risk of breast cancer approaches 90 % for both genes, while the risk of ovarian cancer is 54% for *BRCA1* and 28% for *BRCA2* [Antoniou et al., 2003; Antoniou et al., 2010]. Although these discoveries have shed valuable insights into understanding breast cancer susceptibility in high-risk families, highly penetrant mutations in these genes account for only a small fraction of all breast cancers [Easton et al., 1999; Pharoah et al., 2002]. It has also been suggested that breast cancer risk may be influenced by multiple loci with modest effects [Antoniou et al., 2001; Pharoah et al., 2002]. Given the indisputable link between *BRCA1* and *BRCA2* and hereditary breast cancer, it is important to evaluate whether more modestly penetrant variants in these genes might contribute to sporadic breast cancer risk.

Appropriate medical follow-up, including early and more frequent mammography and pelvic examinations for the early detection of ovarian cancer, is therefore essential for women carrying mutations in these genes. At the moment, the emphasis is on early detection; preventive measures are mostly limited to prophylactic surgery, most notably annexectomy in post-reproductive women to reduce the risk of both ovarian and breast cancer. Clinical molecular genetic testing will continue to grow in importance for patient management. Genetic tests provide valuable information to confirm diagnoses, assess patients’ risks of developing diseases, and select appropriate therapies [Coughlin et al., 1999; Narod et al., 2000]. Because mutations in both of these genes are distributed throughout the loci, accepted clinical protocols involve screening their entire coding regions [Eng et al., 2001]. Currently, most laboratories performing diagnostic analysis of the *BRCA* genes proceed to systematic and complete gene sequencing, using PCR of exons and intron-exon boundaries coupled to a pre-screening step to identify anomalous amplicons.

BRCA mutational spectrum has not been entirely characterized. More than half of the mutations cause the lost 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 over 3500 genetic variants *BRCA1* and *BRCA2* are reported as having uncertain clinical significance [BIC database]. A variety of approaches including theoretical translation to identify termination codons, biochemical assays to assess protein function and RNA splicing, and population and family studies to associate variants with disease, are all employed to define the clinical risk associated with genetic variants. Although the specific criteria to classify genetic variants differ between laboratories, they basically can be classified as deleterious mutations, variants of uncertain clinical significance, and benign polymorphisms. One can further subdivide the uncertain variants to include two additional groups: favor SNPs (unlikely to convey cancer risk) and suspected deleterious (likely to convey cancer risk) [Judkins et al., 2004].

It is well established that rare mutations in *BRCA2* predispose to familial breast cancer, but whether common variants at this locus contribute more modest risk to sporadic breast cancer has not been thoroughly investigated. Common Single Nucleotide Polymorphisms (SNPs) are usually present in human genome, defining alleles whose frequencies widely vary in different populations. Either intragenic or intronic, silent or generating aminoacid substitutions, SNPs cannot be afforded themselves a predisposition status. However, prevalent SNPs can be used to define gene haplotypes, with also various frequencies. *BRCA1* alleles are described almost completely by 10 canonical haplotypes derived from 14 prevalent single nucleotide polymorphisms (SNPs) that are detected during most genetic tests [Judkins et al., 2003; Judkins et al., 2005]. This could also be the case for *BRCA2* [Freedman et al., 2004]. Since some mutation can easily be assigned to haplotypes, SNPs can therefore provide usual information in interpreting gene mutations effects on hereditary predisposition to cancer. *BRCA2* SNPs can be easily detected by entire gene sequencing, which is widely used in predisposition diagnosis. Here we describe 10 *BRCA2* SNPs identified by complete gene sequencing.

MATERIAL AND METHODS

26 patients from 17 HBOC families were recruited at the Sf. Spiridon University Emergency Hospital of Iaşi, Romania. Index cases were diagnosed with breast (13) and ovarian (2) cancers. Breast cancers included 7 early-onset cases (< 40 years), one male breast cancer, and two bilateral cases. Median age at diagnosis of breast cancer was 40 years. Two multiple cancer cases (ovarian/stomach and breast/ovarian) were studied. Nine additional patients from these families were screened for presence of *BRCA* mutations. All patients agreed by written informed consent. Personal and familial cancer histories were obtained from patients and some participating relatives, in order to draw familial anamnesis for the disease.

Genomic DNA was extracted from 10 ml peripheral blood using the Wizard™ Genomic DNA purification kit (Promega Inc, Madison, WI, USA). DNA amount was estimated by spectrophotometry. Several pre-screening methods were used to pre-screen for known and unknown *BRCA* mutations, as described elsewhere [Negura et al., 2011].

The entire coding sequence of *BRCA2* gene, including exon/intron boundaries, was analysed using amplification and Sanger sequencing. Polymerase chain reaction amplifying *BRCA* exons were performed in a final volume of 20 µl containing 0.4mM each dNTP, 0.8 µM of each primer (sequence available on demand), 100 ng genomic DNA, and one unit of either ApliTaq® or AmpliTaq®Gold Polymerase with appropriate 1X Buffer (Applied Biosystems Inc, Foster City, CA, USA). PCR cycling comprised an initial denaturation step at 94ºC for 5 min followed by 30 cycles of 94ºC for 20 sec, 54ºC for 20 sec and 72ºC for 30 sec, and a final extension of 7 min at 72ºC.

Amplicons were verified by electrophoresis on a 1,3% agarose gel, then purified by ExoSap® enzymatic digestion (Affymetrix Inc, USA), 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, Madison, WI, USA) and the CEQ8000 Investigator (Beckman Coulter) softwares.

Mutation presence was systematically confirmed by forward and reverse sequencing on a second independent blood sample. All sequence variants are described according to HUGO approved systematic nomenclature. The nomenclature for BIC traditional mutations is also indicated. NCBI SNP database were used to define SNPs

RESULTS AND DISCUSSION

When completely sequencing *BRCA2* gene, various sequence variants were observed, either in a homozygous or heterozygous context. Two *BRCA*2 deleterious mutations, one of them being novel, have been reported previously [Negură et al., 2010]. Two unclassified sequence variants were also described, with one of them being suggestive of a deleterious mutation, as discussed elsewhere [Negură et al., 2010; Negură et al., 2011].

We observed 10 other sequence variants, distributed along exonic regions or within introns. Since *in-silico* analysis systematically showed a harmless status for those variants, we verified their presence in the NCBI-SNP database. All 10 SNPs were previously described and have NCBI-SNP codes, as one can observe in table 1. We defined our SNPs with low caps (a,c,e,f,i,m,j,n,o,p) for an easier approach. Only 4 of the SNPs have been observed either in homozygous and heterozygous context. Figures 1-10 present the SNPs we identified.

Table 1. BRCA2 SNPs observed in our population

BRCA2

Cod polimorfism	Poziţia nucleotidică (Referinţa U14680)	Exon	Codon	Descriere (BIC)	Nomenclatură HGVS	Efect asupra proteinei	Cod NCBI	Scor Grantham	Tip	Numărul de apariţii	Frecvenţa
a	7470	14	2414	7470A>G	c.7242A>G	Ser2414Ser	rs1799955	-	silenţios	15	0,29
c	203	2	5'-UTR	203G>A	c.1-25G>A	-	rs1799943	-	5'-UTR	13	0,25
e	3624	11-05	1132	3624A>G	c.3396A>G	Lys1132Lys	rs1801406	-	silenţios	19	0,37
f	4035	11-06	1269	4035T>C	c.3807T>C	Val1269Val	rs543304	-	silenţios	11	0,21
i	1693	10B	466	1693A>G	c.1366A>G	Ser466Ser	rs1801439	-	silenţios	1	0,02
m	1093	10A	289	1093A>C	c.865A>C	Asn289His	rs766173	68	mis-sens	1	0,02
j	2457	11-02	743	2457T>C	c.2229T>C	His743His	rs1801499	-	silenţios	1	0,02
n	1342	10B	372	1342A>C	c.1114A>C	Asn372His	rs144848	68	mis-sens	9	0,17
o	17IVS-14	intron	-	17IVS-14T>C	c.8034-14T>C	-	hCV3070421	-	intronic	19	0,37
p	21IVS-66	intron	-	21IVS-66T>C	c.8983-66T>C	-	rs4942486	-	intronic	4	0,08

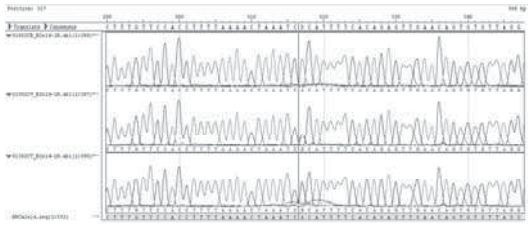

Figure 1. The BRCA2-a Polymorphism (c.7242A>G) in exon 14, in homozygous G (low) and heterozygous A/G (middle) forms, compared to the wild-type homozygous A form (up) and the reference sequence.

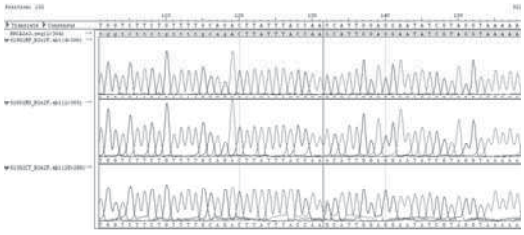

Figure 2. The BRCA2-c Polymorphism (c.1-25G>A) in exon 2, in homozygous A (middle) and heterozygous G/A (low) forms, compared to the wild-type homozygous G form (up) and the reference sequence.

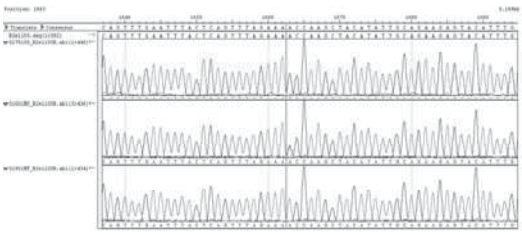

Figure 3. The BRCA2-e Polymorphism (c.3396A>G) in exon 11-05, in homozygous G (up) and heterozygous A/G (middle) forms, compared to the wild-type homozygous A form (low) and the reference sequence.

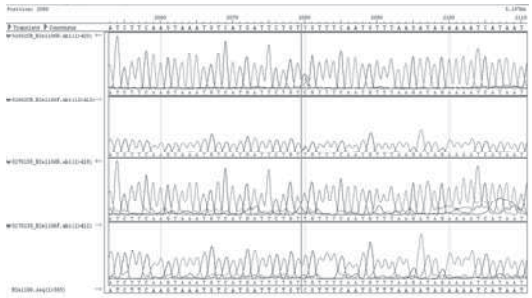

Figure 4. The BRCA2-f Polymorphism (c.3807T>C) in exon 11-06, in heterozygous T/C (up – forward and reverse) form, compared to the wild-type homozygous T form (low – forward and reverse) and the reference sequence. No homozygous C form was observed for this SNP in our population.

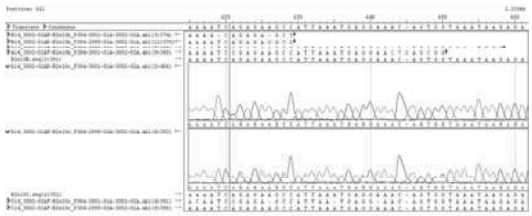

Figure 5. The BRCA2-i Polymorphism (c.1365A>G) in exon 10B, in heterozygous A/G (up) form, compared to the wild-type homozygous A form (low) and the reference sequence. No homozygous G form was observed for this SNP in our population.

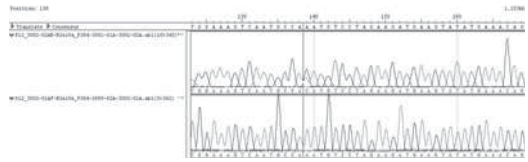

Figure 6. The BRCA2-m Polymorphism (c.865A>C) in exon 10A, in heterozygous A/C (up) form, compared to the wild-type homozygous A form (low) and the reference sequence. No homozygous C form was observed for this SNP in our population.

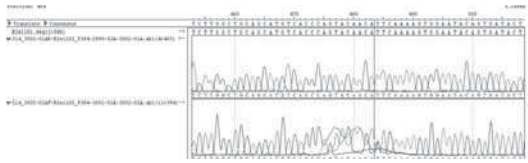

Figure 7. The BRCA2-j Polymorphism (c.2229T>C) in exon 11-02, in heterozygous T/C (low) form, compared to the wild-type homozygous T form (up) and the reference sequence. No homozygous C form was observed for this SNP in our population.

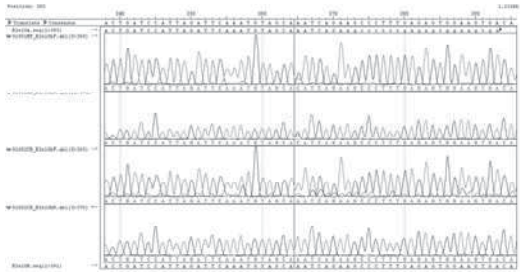

Figure 8. The BRCA2-n Polymorphism (c.1114A>C) in exon 10B, in heterozygous A/C (up – forward and reverse) form, compared to the wild-type homozygous A form (low – forward and reverse) and the reference sequence. No homozygous C form was observed for this SNP in our population.

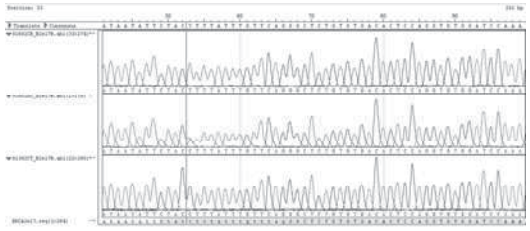

Figure 9. The BRCA2-o intronic Polymorphism (c.8034-14T>C), in homozygous C (low) and heterozygous T/C (middle) forms, compared to the wild-type homozygous T form (up) and the reference sequence.

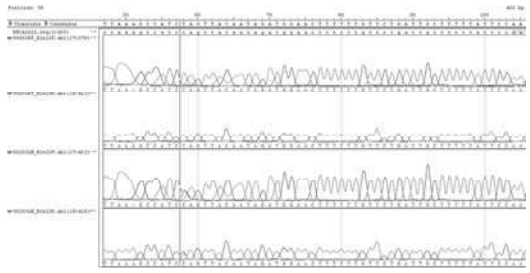

Figure 9. The BRCA2-p intronic Polymorphism (c.8983-66T>C), in heterozygous T/C (up – forward and reverse) form, compared to the wild-type homozygous T form (low – forward and reverse) and the reference sequence. No homozygous C form was observed for this SNP in our population.

CONCLUSIONS

Of the 10 SNPs described, two are intronic, one is in the 5’-UTR of the gene, while 7 are distributed along the exons. Exons 11 and 10, each with 3 SNPs, are the most affected ones, which is not surprising in view of their size. Of the 7 exonic SNPs, 5 are silent on protein sequence, while 2 cause aminoacid substitutions (Asn289His and Asn372His – Grantham score 68), apparently without perturbing the protein general structure and functions. Further studies on haplotyping *BRCA*2 gene on the base of described SNPs are under way.

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