Identification of a recurrent BRCA1 mutation in two breast/ovarian cancer predisposition families with distinct phenotypes, by using allele-specific multiplex-PCR

Identificarea unei mutații recurente BRCA1 în două familii cu predispoziție la cancer mamar/ovarian cu fenotipuri distincte, prin PCR-multiplex alelă-specific

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Abstract

Background. *Hereditary predisposition to breast and ovarian cancer is mainly attributable to predisposition genes BRCA1 and BRCA2. Lifetime risk of developing either breast or ovarian cancer is significantly higher for BRCA germ line deleterious mutation carriers compared to the general population. Screening for BRCA mutations is nowadays standard practice in the western world, and allows medical follow-up and genetic counseling for patients. In spite of the large BRCA mutation spectrum, some recurrent mutations are responsible for a large percentage of predisposition families. In Eastern European populations, BRCA1 5382insC is a common recurrent Ashkenazi founder mutation.* ***Patients and methods.*** *We identified and recruited 19 hereditary breast and ovarian cancer (HBOC) families, with at least 3 cases of epithelial breast or ovarian cancer within the same family line. All patients agreed by written informed consent. DNA was extracted from peripheral blood. A particular allele-specific multiplex-PCR method was used to screen for the recurrent 5382insC mutation. Amplification and Sanger sequencing were used to confirm the presence of the mutation.* ***Results.*** *The BRCA1 5382insC mutation was found in two different HBOC families, with different breast/ovarian cancer familial history and without any apparent degree of relatedness.* ***Conclusions.*** *We demonstrated the feasibility of a rapid screening in our population for known recurrent BRCA1 mutations, by using simple PCR-based techniques. This result, the first one in Romania, could open the way for a population study to determine the frequency of 5382insC in the Romanian population. This could also develop the oncogenetic approach and follow-up of BRCA mutations bearers in Romania.*

Keywords: *hereditary breast/ovarian cancer (HBOC), BRCA recurrent mutation, allele-specific multiplex-PCR*

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Rezumat

Predispoziția ereditară la cancerul mamar şi la cancerul ovarian este atribuită cu precădere genelor de predispoziție BRCA1 şi BRCA2. Riscul de a dezvolta cancer mamar sau cancer ovarian pe toată durata vieții este cu mult mai mare la purtătorii de mutații germinale deletere BRCA, comparativ cu populația generală. Screeningul mutațiilor BRCA este la ora actuală o practicăî standardizată în lumea occidentală, permiţînd monitorizarea medicală şi sfatul genetic acordat pacienților. Cu toate că spectrul mutațiilor BRCA este deosebit de larg, unele mutații recurente sunt responsabile de un procent important al familiilor cu predispoziție. În populațiile est-Europene, BRCA1 5382insC este o mutație comună, recurentă, cu effect fondator Ashkenazi. ***Pacienți şi metode.*** *Am identificat şi recrutat 19 familii cu predispoziție ereditară la cancer mamar şi ovarian (familii HBOC), cu cel puțin 3 cazuri de cancer epithelial mamar sau/şi ovarian în aceeaşi linie familială. Toți pacienții au acceptat participarea prin semnarea unui consimțământ informat. ADN a fost extras din sângele periferic. O metodă particulară de PCR-multiplex alelă-specific a fost folosită pentru a căuta mutația recurentă 5382insC. Amplificarea şi secvențierea Sanger au fost utilizate pentru a confirma prezența mutațiilor.* ***Rezultate.*** *Mutația BRCA1 5382insC a fost identificată în două familii HBOC diferite, cu istoric familial diferit de cancere mamare/ovariene şi fără nici un grad evident de înrudire.* ***Concluzii.*** *Am demonstrat fezabilitatea unui screening rapid în populația noastră pentru identificarea de mutații cunoscute recurente, prin tehnici simple bazate pe PCR. Acest demers, primul în România, ar putea deschide calea către un studiu populațional pentru determinarea frecvenței mutație 5382insC în populația României. De asemenea, ar permite dezvoltarea în România a demersului oncogenetic şi a monitorizării purtătorilor de mutații BRCA.*

Cuvinte cheie: *cancer mamar/ovarian ereditar (HBOC), mutații recurente BRCA, PCR-multiplex alelă-specific.*

Introduction

Breast cancer is the most common cancer in women worldwide, including Romania, where its incidence seriously increased during the last decade [1,2,3]. Ovarian cancer is the fourth cause of cancer mortality [4]. BRCA1 [5] and BRCA2 [6] are major cancer predisposition genes, responsible for a large percentage of hereditary breast and ovarian cancer (HBOC) families. Germinal mutations in BRCA1 gene are responsible for about 45% of families with increased incidence of breast cancer and for over 80% of families with significantly increased incidence of both early-onset breast and ovarian cancer [7]. Screening for mutations in these two genes is now standard practice for HBOC cases in Western Europe and North America, and allows medical follow-up and genetic counseling adapted to the needs of individuals in such families. The consequences of germ-line mutation of either of the BRCA genes are serious: by 50 years of age, 45% of BRCA1 carriers and 20% of BRCA2 carriers will have already developed breast cancer, compared to about 3% of non-carriers [8].

BRCA mutational spectrum has not been entirely characterized. Over one thousand small sequence variations have been reported in the Breast Cancer Information Core (BIC) database [9]. More than half of these mutations (over 300 in BRCA1 and 200 in BRCA2) cause the loss of function by premature protein synthesis termination [10], and around 60% are unique to a family [9]. In Eastern Europe, three BRCA1 known recurrent mutations (300T>G, 185delAG and 5382insC) are responsible for a majority of HBOC families and are either involved in cancer cases or may be detected in healthy individuals from HBOC or non-HBOC families [11]. These three mutations were previously described as Ashkenazi Jewish founder mutations, also found frequently in general population [12,13]. Several Eastern European population studies revealed a high frequency of the 5382insC mutation [14,15,16,17,18]. Several rapid and cheap pre-screening methods were developed for the detection of 5382insC. In the

following we describe the implementation, optimization and use of a particular allele-specific multiplex-PCR method [19].

Patients and methods

HBOC families were recruited on the basis of the following criteria:

- when three of more breast or ovarian cancer cases were diagnosed within the same family line;
- situations with less than three cancer cases but diagnosed before the age of 40;
- breast and ovarian cancer cases in the same family;
- breast cancer in men;
- bilateral breast cancer;
- medullary type of breast cancer.

17 HBOC families were identified at the "Sf. Spiridon" University Emergency Hospital of Iaşi, Romania, and 26 members, index cases when possible, were recruited from these families. 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 collected on heparin anticoagulant, by optimization of the WizardTM Genomic DNA purification kit (Promega Inc, Madison, WI, USA). Two 5 ml samples were processed in parallel, and DNA was eluted in 1 ml TE Buffer. After appropriate dilutions in 50 µl, DNA amount was estimated by spectrophotometry, using the DU800 spectrophotometer (Beckman Coulter Inc, Fullerton, CA, USA). Allele-specific multiplex-PCR was optimized and performed for detection of the recurrent 5382insC BRCA1 mutation, using the 3-primers multiplex-PCR as reference method [Chan 1999]. PCR was finally performed in a final volume of 50 µl, using variable quantities of reagents following optimization steps. The amplifying program was also optimized alternatively on thermocyclers as MastercyclerTM Gradient (Eppendorf AG, Hamburg, Germany) or PalmCyclerTM (Corbett LifeSciences / Qiagen, Germantown, MD, USA). Each PCR reaction consisted of an initial denaturation of 10 min at 95°C, followed by 35 cycles of 15 s of denaturation at 94°C, 15 s of annealing at 57°C, and 30 s of extension (with an increment of 1 s for each subsequent cycle) at 72°C, and a final extension step of 5 min at 72°C. Amplification aliquots were migrated, using the Sub-Cellp System for Submerged Horizontal Electrophoresis (Bio-Rad Laboratories Inc, Hercules, CA, USA) at 5 V/cm on 2% or 3% agarose gels containing 0,5 µg ethidium bromide for 1 ml gel solution. Following electrophoresis, gels were visualised under UV in a G:BOX Chemi™ Gel Documentation System (Syngene, Cambridge, UK) and interpreted with GeneSnap™ and GeneTools™ software.

Principle of the method

Several recurrent BRCA1 mutations (185delAG, 5382insC, or 300T>G) are proved to be responsible for a majority of HBOC families in Eastern European populations. The5382insC Ashkenazi founder mutation represents an insertion of a C nucleotide within BRCA1 exon 20. In the HUGO approved systematic nomenclature [20], where nucleotide numbering starts with A of initiator ATG in position 1, the mutation is generating a frameshift in codon 1756 (CAG), coding for glutamine 1756. C mononucleotide insertion is changing the codon in CCA, coding for a proline 1756. Rather rare as amino acid, proline could have major implications on the general conformation of the protein. Moreover, the frameshift makes the following 72 amino acids to be aberrant, with an opale stop codon downstream, truncating the protein of its 35 last C-terminal residues. Overall, 107 C-terminal amino acids of BRCA protein are either missing or abnormal. The C-terminus of BRCA1, besides containing a BRCT essential domain, is responsible for interactions with BRCA2, transcriptional cofactor p300/CBP, with RNA Polymerase II or

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68161 gtgatctgcc tgcctcagtc tcccaaagtg ctaggattac aggggtgagc cactgcgcct
68221 ggcctgaatg ccttaaatat gacgtgtctg ctccacttcc attgaaggaa gcttctcttt
68281 ctcttatcct gatgggttgt gtttggtttc tttcagcatg attttgaagt cagaggagat
68341 gtggtcaatg gaagaaacca ccaaggtcca aagcgagcaa gagaatccca ggacagaaag
68401 gtaaagctcc ctccctcaag ttgacaaaaa tctcacccca ccactctgta ttccactccc
68461 ctttgcagag atgggccgct tcattttgta agacttatta catacataca cagtgctaga
68521 tactttcaca caggttcttt tttcactctt ccatcccaac cacataaata agtattgtct
68581 ctactttatg aatgataaaa ctaagagatt tagagaggct gtgtaatttg gattcccgtc
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Figure 1. Genomic context of BRCA1 exon 20 (bold), with the location of 5382insC duplication after a series of three other C (underlined)

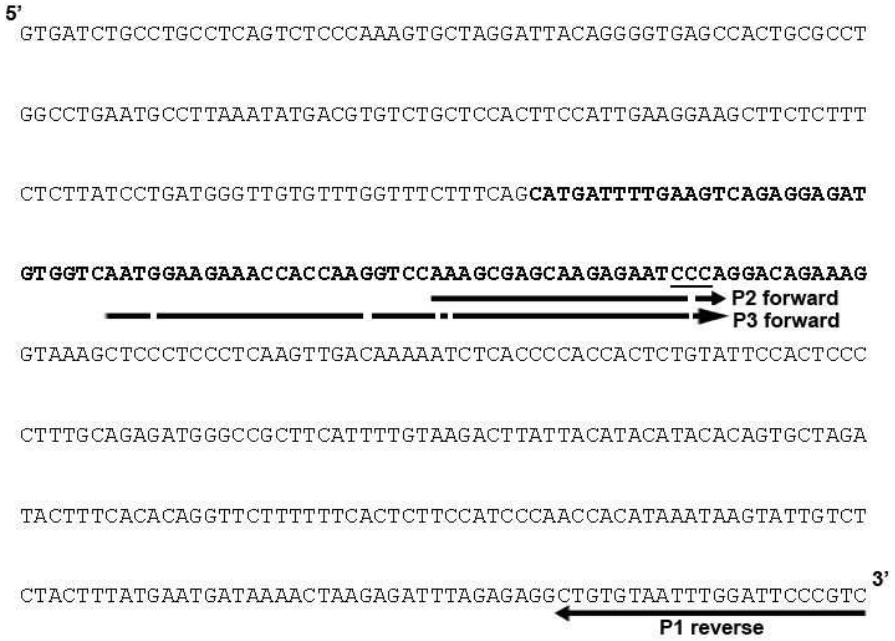

Figure 2. Position of respective primers on BRCA1 exon 20 FASTA sequence

RNA Helicase A, with transcriptional co-repressor CtIP, and also with HDAC proteins 1 and 2 involved in histone deacetylation and chromatin remodeling. Thus, deletion of this terminal domain of the protein has major consequences on cell metabolism. According to the BRCA1 coding reference sequence U14680 [21], the inserted C nucleotide arises in position 5266, after a succession of three other cytosines, hereby representing rather a duplicated C then an insertion. Hence, the correct event happening is in fact the c.5266dupC, with a p.Gln1766ProfsX73 effect on BRCA protein, according to NP_009227.1 reference sequence [21]. Still, the obsolete nomenclature system, actually widely used in ancient literature and by mutation databases as the BIC [9], uses to start numbering 119 nucleotides 5'UTR upstream the initiation ATG codon, therefore considering this mutation as an insertion in position 5382. As a convention, we will systematically refer to HUGO nomenclature, using NCBI Genbank reference sequences U14680 for coding sequence, NC_000017.10 for genomic sequence and for protein sequence. Nevertheless, we will keep the 5382insC appellation as overwhelming literature data is using it.

In order to quickly screen for 5382insC heterozygous presence in our HBOC patients, we chose a previously described 3 primers mul-

tiplex-PCR method [19]. In the BRCA1 Genbank sequence below (*Figure 1*, issued from NC_000017.10 genomic sequence), bold letters represent exon 20 (5313-5396 positions in U14680), flanked by 156 5'- and 240 3'- intronic nucleotides. The three C nucleotides preceding the duplication are underlined capital letters. Our mutation represents the duplication of the last C, in 5385 position (BIC system) or 5266 position (HUGO system).

For the detection of the mutation, three primers are chosen for a multiplex-PCR (*Figure 2*), with a common reverse primer (P1), one forward wild-type specific (P2) and one forward mutation specific (P3) primers. The competing primers, P2 and P3, differ in length by 23 nucleotides, allowing for easy discrimination of PCR products by classical electrophoresis. The 3 primers [19] were verified in the NIH database using BLAST application against false hybridization. Also, NetPrimer software was used to verify possible primer dimers or cross-dimers generation. Primer sequences were as following, with modified (mismatch) nucleotides underlined.

Primer 1 (reverse) :
5' GACGGGAATCCAAATTACACAG 3', common;

Primer 2 (forward) : **5'**
AAAGCGAGCAAGAGAATCGCA 3' , wild-type specific

Primer 3 (forward) : **5'**
AATCGAAGAAACCACCAAAGTCCTTAGCGAGCAAGAGAATCACC 3', mutation (5382insC) specific

The forward P2 and P3 primers deliberately contain one mismatch nucleotide cole ? to their respective 3' end. Although this kind of oligonucleotide constructions generally hybridizes and amplifies slower (the aim of primers is actually specificity), during the early cycles of amplification the "wrong" mismatched sequences generate mutagenized PCR products that are refractory to cross-amplification by the competing primer, thereby ensuring specificity of the reaction and allelic discrimination for heterozygote cases. The long (mutant) primer (P3) also incorporates two additional mismatched bases at two contiguous positions corresponding to the 5' end of the short (wild-type) primer. During the final cycles of the PCR reaction, heteroduplexes may be formed from the short and long products, but the contiguous mutagenized sequences in the long product prevent filling up of the short product by using the long strand as template. As a result, the mutant and wild-type products are separated mutagenically.

When using all three primers in the same reaction, two distinct products can be expected: one 271 base pair fragment, wild-type specific, amplified by P1 with P2, and one 295 base pair fragment, mutation-specific, amplified by P1 with P3. Thus, a normal wild-type patient will generate only the 271 bp fragment from both alleles, whereas a heterozygous 5382insC patient will generate both 271 and 295 bp fragments.

Results

The optimization steps comprise all experiences requisite in order to prepare an efficient final protocol, adapted to local equipment and working conditions. Each time a new technique is implemented, or when researching background changes, each research step requires a deepened and recurring analysis in order to establish an optimal efficiency of all reaction parameters within the new conditions. Each PCR reaction has its own optimal parameters, but times and often these parameters are comprised within functional intervals. Inside such an interval, each parameter may slightly vary without perturbing the global efficiency of the reaction; therewith, modifying simultaneously several parameters within the limits of the functional interval can shift the whole reaction to failure. Time and again, researchers who do not manage to make a functional PCR reaction locate at a borderline of the functional interval, where one only parameter should be changed; modifying any other parameter pushes the reaction far away from the optimal condi-

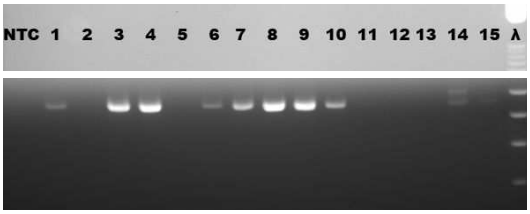

Figure 3. Initial amplification of the region of interest by the 3-primers mutiplex-PCR method
(NTC = No template control; λ = 50 bp step ladder)

tions. This problem is even more complex when using multiplex-PCR, with different functional intervals for each amplification, and with global parameters which have to comply with all involved reaction parameters. During the optimization of our 3-primers multiplex-PCR reaction, we managed to find out the optimal conditions for each of the following parameters, varying the parameters recommended by the original authors [Chan 1999].

In *Figure 3,* the first 3-primers amplification assay is presented, with PCR conditions recommended by authors [19], i.e. 25 ng of genomic DNA, 10 μL of reaction mixture consisting of 1μL PCR reaction buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 0.01 g/L gelatin), 3.25 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, and 50 kU/L TaqPolymerase). Primers were added for a concentration of 0.12 mmol/L each. – de reformulat In our case, we used 1,25 units of GoTaq Polymerase (Promega) in appropriate 1X buffer for each reaction of 50 μL. In figure 3, one can observe the 271 bp fragment apparition in lanes 1, 3, 4, 6, 7, 8, 9 and 10 (different patients). Two less intense bands are visible in lanes 14 and 15, which could theoretically correspond to 271 and 295 bp fragments in heterozygous individuals. Still, at this level the method has poor resolution and cannot be used in diagnosis. Amplification in lanes 2, 5, 11-13 was not successful.

In order to surpass this decisional problem, we tried to ameliorate the resolution by optimizing reaction conditions. Thus, we used 100-150 ng genomic DNA per reaction (4-6-fold higher then recommended), because lesser quantities generally generate little UV signal

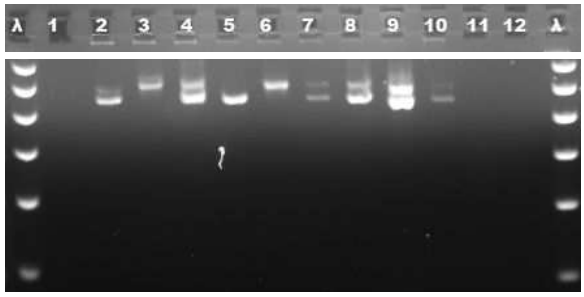

Figure 4. Amplification with the 3-primers mutiplex-PCR method, by varying different reaction parameters as explained in text
(λ = 50 bp step ladder)

within our routine PCR protocols. Also, primers were added in equivalent concentrations of 0,2 μM (1μl solution 0,01 mM per reaction), meaning the double of the initial concentration. Magnesium salt concentration in the reaction solution was reduced from 3,5 to 3 mM (6 μl used from a 25 mM stock solution). *Figure 4* shows the results of all mentioned modifications. Lane 1 corresponds to a no template control, proving lack of contamination. Lanes 2 and 5 correspond to a two-primer (P1 and P2) amplification of normal allele, with a 271 bp amplification product; in lane 2 we used 150 ng genomic DNA, whilst only 100 ng gives better amplification results in lane 5. Lanes 3 and 6 are corresponding to the same genomic templates, but amplifications used primers P1 and P3, so the mutated allele (we obviously used a 5382insC patient, detected in lane 14 figure 3) generates a 295 bp fragment. In lanes 4 and 7, we cumulated all 3 primers for the detection of both alleles. We can easily point out that 150 ng genomic DNA allows a better amplification of both alleles (lane 4) then 100 ng (lane 7), although two-primer amplifications worked better with 100 ng (lanes 5 and 6) then with 150 (lanes 2 and 3). Lane 8 repeats amplification of lane 4, while in lane 9 we used a double primer concentration (0,4 μM), and in lane 10 we used 300 ng template DNA. Modifying differently the reaction parameters (lanes 11 and 12) "killed" all amplifications.

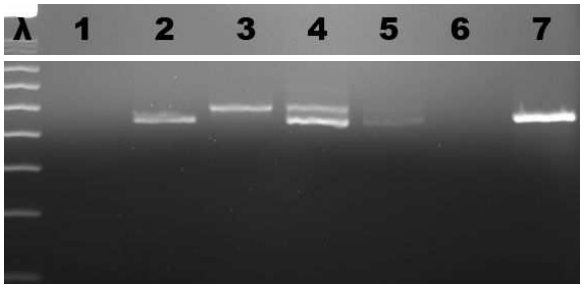

Figure 5. Detection of heterozygous 5382insC mutation with 3-primers mutiplex-PCR method
(λ = 50 bp step ladder)

Two main conclusions can be drawn out from figure 4. On one hand, more primer means better amplification, as one can notice lane 9. Therefore, the better amplifications work, the harder is to separate the two amplification products, which could lead to false negatives. Bands are more intense in lane 9, whereas bands are better separated in lane 4. Certainly, we could imagine a separation system with better resolution, as a 4% agarose gel or a PAGE; we could also imagine a diminution of cycle number in amplification program. Still, the best thing to imagine remains to keep primers at 0,2 μM, considering lane 4 the better compromise between intensity and resolution.

On the other hand, more template DNA means worse amplification. We certainly did ameliorate reaction efficiency by rising DNA quantity from 100 to 150 ng (*Figure 4*, lanes 4 and 7); we definitively destroyed efficiency by raising this quantity to 300 ng (lane 10). This is a striking evidence of a borderline situation in optimizing PCR reaction, and an argument for the "better, the enemy of good" concept.

The maximum resolution we obtained for this technique is presented in figure 5. Lane 1 contains a no template control. Lane 2 contains the 271 bp fragment obtained with P1 and P2 primers on normal allele, whereas in lane 3 one can observe the 295 bp fragment obtained with P1 and P3 on mutated allele. In lanes 4 and 5, two different patients, heterozygous for 5382insC, were amplified with all three primers; both fragments were obtained, for both alleles. In lane 7, a normal wild-type patient generates on only, twice intense, 272 bp band corresponding to 2 normal alleles (*Figure 5).*

As a main conclusion, the optimized method for detection of BRCA1 5382insC heterozygous mutation by allele-specific multiplex-PCR works properly. It is a cheap, robust and efficient method which can rapidly discriminate between wild-type homozygous and heterozygous individuals. We consider therefore this method as appropriate for rapid screening of 5382insC in large population lots. As for diagnosis, the method described should only play a pre-screening role, and should constantly be confirmed by DNA sequencing of BRCA1 exon 20.

We analyzed by the method mentioned above a lot of 26 patients from 17 HBOC families recruited in North-Eastern Romania. Interesting, from a scientific point of view, is that we identified 5382insC mutation in three patients from two distinct unrelated families, with totally different cancer phenotypes. In family 006 (*Figure 6*), a breast cancer predisposition family, one can notice the presence of 5 breast cancer cases, bilateral or multiple cases, together with other cancers, such as lung or rectal cancer. Recruited patients (indicated by arrows) developed breast cancer at age 49 and 36, an example of genetic anticipation. On the contrary, family 007 (*Figure 6*) may be considered an ovarian cancer predisposition family, with an agglomeration of 5 ovarian cancer cases and only one breast cancer. The recruited patient developed ovarian cancer at age 50 and stomach cancer at age 55. In fact, stomach cancer and 5382insC mutation are the only common points between these two unrelated families. The difference in familial phenotypes, whether caused by environmental factors or by other unknown genetic difference, remains unclear.

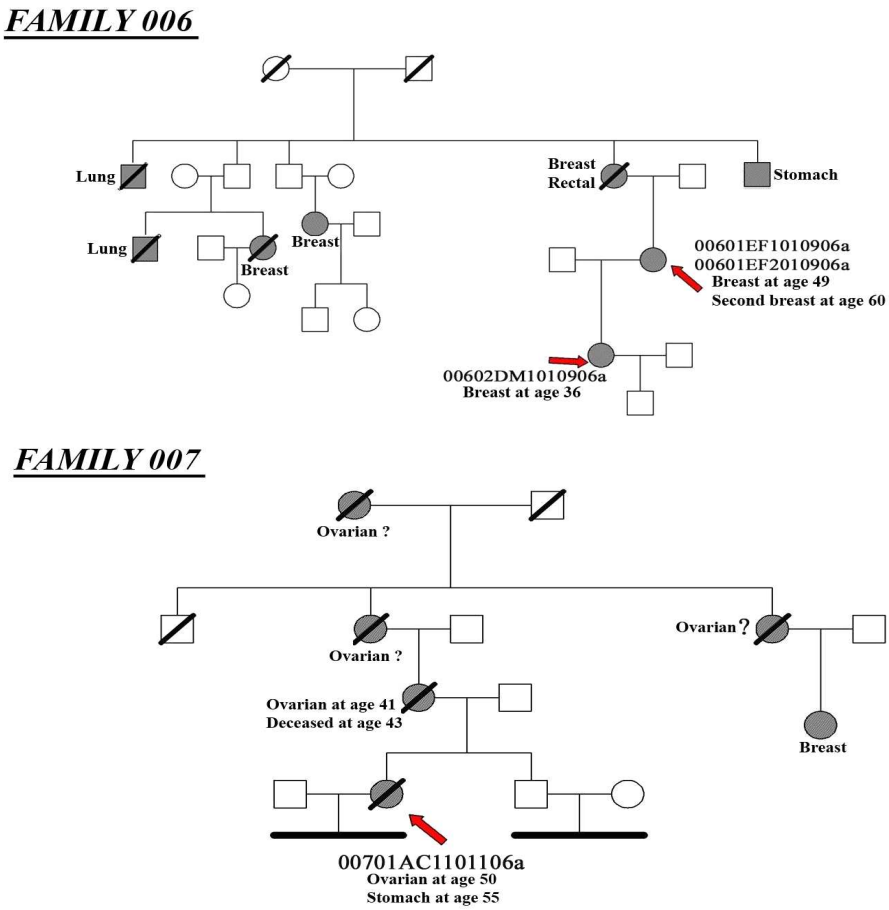

Figure 6. Detection of 5382insC mutation in two unrelated HBOC families

Conclusions

By optimizing an allele-specific multiplex-PCR method, we searched for recurrent 5382insC BRCA1 mutation in 26 recruited patients from 19 HBOC families. The BRCA1 5382insC mutation was found in two different HBOC families, with different breast/ovarian cancer familial history and without any apparent degree of relatedness. Sanger sequencing confirmed the presence of the mutation. We demonstrated the feasibility of a rapid screening in our population for known recurrent BRCA1 mutations, by simple PCR-based techniques. This outcome, the first one in Romania, could open the way for a population study to determine the frequency of 5382insC in the Romanian population. This could also develop the oncogenetic approach and follow-up of BRCA mutations bearers in Romania.

Acknowledgements

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Conflicts of interest

The authors declare that there are no conflicts of interest concerning this paper.

Abbreviations

BRCA1/2 – Breast Cancer Gene 1/2
PCR – Polymerase Chain Reaction
HBOC – Hereditary Breast and Ovarian Cancer
BIC – Breast cancer Information Core database
HUGO – Human Genome Organization
BRCT – BRCA C-terminal domain
HDAC – Histone Deacetylase
UTR – Untranslated Region
NCBI – National Center for Biotechnology Information
NIH – National Institute of Health
BLAST – Basic Local Alignment Search Tool
PAGE – Polyacriylamide Gel Electrophoresis
CNCSIS – Consiliul Național al Cercetării Științifice din Învățământul Superior

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