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BRCA1 5382insC founder mutation has not a significative recurrent presence in Northeastern Romanian cancer patients

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Abstract

Developed two decades ago, oncogenetic medical practice mainly concern breast, ovarian and colorectal cancers, and is targeting the hereditary risk factor, the only one that shows positive predictive value justifying the molecular diagnosis. Screening for *BRCA1* and *BRCA2* gene mutations is standard practice today for hereditary breast and ovarian cancer (HBOC) families in developed countries, offering the possibility of medical follow-up. The gold standard for molecular diagnosis is Sanger sequencing of all exons and exon-intron boundaries, which is expensive and time consuming. More than 3000 *BRCA* sequence variants are reported in international databases, but in some populations or ethnic groups a few founder mutations showed to have a recurrent presence. This may be very useful in establishing a combined technical approach for mutation detection, including rapid and cheap pre-screening methods for most common mutations. The *BRCA1* 5382insC mutation has an Ashkenazi founder effect and is also the second most recurrent mutation in Eastern European populations, having been already identified in several Romanian HBOC patients. Here we present a complete screening of consecutive series of breast and ovarian cancer patients for the presence of *BRCA1* 5382insC. The presence of the mutation was investigated by allele specific multiplex-PCR on genomic DNA extracted from peripheral blood. No mutation carrier was identified among breast or ovarian cancer patients. Our findings suggest that *BRCA1* 5382insC may not have a strong recurrent effect in Romanian population comparing to neighboring countries. This may be particularly useful in establishing further pre-screening strategies.

Keywords: hereditary predisposition to cancer, *BRCA1* 5382insC mutation, pre-screening, recurrent mutation.

Introduction

Breast cancer is responsible for the majority of deceases due to cancer in women all over the world, covering almost a quarter of all types of cancer [1]. According to *World Health Organization* (*WHO*) [2], breast cancer incidence in the western world attained 96 per 100 000 in 2012, being in continuous increase by 2% every year. In Romania, breast cancer incidence in 2012 was 66 per 100 000, and mortality because of the disease was 21.6 per 100 000 [3, 4]. Breast cancer incidence is sensibly higher in western developed countries compared to less developed, probably due to differences in dietary and reproductive lifestyle but is a matter of fact that incidence in latter countries shows these last years a continuous growth [1, 5]. Ovarian cancer is also among the most common malignant disease in women (incidence rising to 13.6 per 100 000 in 2012), and it can be rapidly fatal, with mortality estimated to 7 per 100 000 [4]. Although the majority of cancers are sporadic, about 5–10% of all breast and 10–15% of ovarian cancer cases can be attributed to genetic hereditary risk factors, being transmitted by Mendelian inheritance of autosomal dominant gene mutations [6]. Inherited predisposition could hereby explain around half of hereditary breast and ovarian cancer (HBOC) aggregation in some families [7].

At least 20 genes have been associated by now with these types of cancer [8, 9], some of them showing high penetrant genetic susceptibility, (*BRCA1*, *BRCA2*, *P53*, *PTEN*, *STK11/LKB1* or *CDH1*), while others are others considered as moderate genetic factors (*CHEK2*, *PALB2*, *BRIP1*, *ATM*, *RAD51C* and others). The most significant and well-characterized hereditary genetic risk factors for HBOC syndrome are germline mutations of the genes *BRCA1* [10] and *BRCA2* [11]. New genes responsible for breast and ovarian cancer are still expected to be identified as many HBOC families are negative for mutations in all above genes.

The consequences of germline mutations in high-penetrance genes are serious, lifetime risk of breast and ovarian cancer being extremely high. Data on age-dependent cancer risk in mutation carriers are various and even controversial [12]. It is generally estimated nowadays that women with an inherited *BRCA1* mutation have up to 80% lifetime risk of developing breast cancer and up to 65% of developing ovarian cancer, while *BRCA2* mutation carriers have a lifetime risk up to 85% for breast cancer and 25% for ovarian cancer [13]. Screening for *BRCA* mutations become standard practice in the western world as part of oncogenetic practice, giving the possibility of efficient monitoring and follow-up of mutation carriers and of their families. Early detection of the disease, as

well as prevention strategies, widely make part of national health primary care services. Thereby, mutation detection technology rapidly evolved in the last decades and adapted to the needs of each gene and population.

More than 3000 *BRCA* sequence variants are reported in international databases such as BIC or UMD [14, 15], the large majority being single nucleotide substitutions. The truncating and frameshift alterations, clearly pathogenic for completely inactivating the protein, account for a minority of variations, and almost 50% of the reported data have an unclear pathogenic status (unclassified sequence variants). In outbreed populations such as Western European or Northern American ones there is a heterogeneous distribution of rare, unique and familial mutations, in such a way that each HBOC family has almost its own mutation [16]. Mutation screening is in those countries almost entirely depending on full gene sequencing, groping for novel mutations at each test. In some inbreed populations, as well as in descendant populations from inbreed ancestors, there is strong evidence that some common mutations appear much more often than others [17]. It is the case of "founder mutations", such as we can found in the majority of Eastern Europe populations. The most frequent Eastern mutations are the Ashkenazi Jewish 185delAG and 5382insC for *BRCA1* and 6174delT in *BRCA2*, as well as the non-Ashkenazi *BRCA1* 300T>G, in this order of frequency.

We decided to screen for *BRCA1* 5382insC mutation in consecutive series of breast and ovarian cancer patients, independently of their family cancer history. This offers the first wide image of a recurrent mutation distribution in Romanian population, and should bring a first answer to the question whether *BRCA1* 5382insC has a founder effect in our population or not.

Patients and Methods

We investigated the presence of *BRCA1* 5382insC mutation is consecutive series of breast (120 patients) and ovarian (50 patients) cancer patients. The selection of the cases was consecutive and independent of any familial cancer aggregation, familial oncologic history, or clinical/histopathological criteria. The patients were identified and recruited at the Regional Oncology Institute of Iassy, Romania, between 2012 and 2013. All patients agreed to participle to the present study by written informed consent. General data was collected from the patients, including the age at diagnosis, cancer histopathological type, number of birth, number of labor, lactation, oral contraceptive, as well as general cancer risk factors linked to lifestyle.

Genomic DNA was extracted from 2 mL of heparin collected peripheral blood, using the Wizard™ Genomic DNA purification kit (Promega Inc., Madison, WI, USA). Two mL samples were processed in parallel by adapted protocol, as previously optimized [18], and DNA was eluted in 500 μL TE Buffer. After appropriate dilutions in 50 μL, DNA amount was estimated by spectrophotometry, using the DU800 spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA). Dilutions at 50 ng/μL were realized for each sample.

Genotyping of *BRCA1* 5382insC mutation was performed by an adapted allele-specific multiplex-PCR, conceived in 1999 [19] and optimized in Romania in 2010 [20]. Briefly, PCR was performed in 50 μL reaction containing 1.25 units of GoTaq Polymerase (Promega) in appropriate 1X no magnesium containing buffer, 3 mM $MgCl_2$ (6 μL used from a 25 mM stock solution), 0.2 μM each primer (*i.e.*, 1 μL of 0.01 mM solution for common reverse, wild-type specific forward and mutant-specific forward), 0.2 mM each dNTP (*i.e.*, 1 μL of a pre-mixed dNTP solution with 10 mM each), and nuclease-free water qsp 48 μL. The PCR mix was completed with 2 μL genomic DNA at 50 ng/μL, *i.e.*, 100 ng DNA for each reaction as previously demonstrated [20]. We also optimized this reaction for a 20 μL PCR volume, as it will be shown below.

Each PCR reaction consisted of an initial denaturation of 10 minutes at 95^0C, followed by 35 cycles of 15 s of denaturation at 94^0C, 15 s of annealing at 57^0C, and 30 s of extension (with an increment of one second for each subsequent cycle) at 72^0C, and a final extension step of 5 minutes at 72^0C. Amplification aliquots were migrated, using the Sub-Cell® System for Submerged Horizontal Electrophoresis (Bio-Rad Laboratories Inc., Hercules, CA, USA) at 5 V/cm on 2% agarose gels containing 0.5 μg ethidium bromide for 1 mL of gel solution. Following electrophoresis, gels were visualized under UV in a Digi Genius Gel Documentation System (Syngene, Cambridge, UK) and interpreted with GeneSnap™ and GeneTools™ software.

The 5382insC mutation was used according to the older BIC database [14] nomenclature, while *Human Genome Variation Society* (HGVS) recommendations [21] denominate the same mutation c.5266dupC. Reference sequence in *GeneBank* for *BRCA1* gene was U14680 [22].

Results

Patients group definition and classification

We recruited for the present study 170 patients with neoplastic pathologies. One hundred twenty patients had breast cancer and 50 patients were diagnosed with ovarian cancer. As mentioned earlier, the selection of the cases was consecutive and independent of any familial cancer aggregation, familial oncologic history, or clinical/histopathological criteria. However, we collected general data from the patients, including the age at diagnosis, cancer histopathological type, number of birth, number of labor, lactation, oral contraceptive, as well as general cancer risk factors linked to lifestyle. This permitted us to have a clearer look over the chosen groups and to further classify them according to these criteria.

As on can see in Figure 1, the distribution of breast cancer cases on age tranches is congruent with age-related incidence worldwide, according to *WHO* [2] and Globocan [1] data. The vast majority of cases developed the disease between ages 50 and 70, while relative few cases are reported as early onset. Surprisingly, the situation is quite different for ovarian cancers. Worldwide incidence is age-dependent and increase with every 10-years age tranches, stabilizing after 60 years, which means that the majority of ovarian cancer cases in a population should concentrate beyond the age of 60 [23]. The situation in our population group is slightly different (Figure 2), with a vast majority of cases being diagnosed between ages

30 and 60. We can even point out that the vast majority of ovarian cancers in our population were diagnosed before 60 years, almost 2/3 of the cases are reported before 50 years, and around 1/4 before the age of 40 years. All these data emphasize a very young ovarian cancer group, much younger than in western world or even neighboring countries. There is lack of data provided for age-dependent incidence of ovarian cancer in Romania, but our findings should bring the attention on the particularities of ovarian cancer in our population, and therefore for a much intensive preoccupation for oncogenetic counseling and molecular testing addressed to ovarian cancer patients.

In Figures 3 and 4, we present the distribution of breast (Figure 3) and ovarian (Figure 4) cancer cases, according to the histopathological type. Our data are in accord with literature statistics [1, 2], which mean a large majority of ductal carcinoma for breast cancer and more than half of adenocarcinoma for ovarian cancer cases. A further subdivision of ductal carcinoma reveals a 2/3 majority of invasive cases, followed by invasive breast cancer of no special type (NST) and breast cancer not otherwise specified (NOS). As a notable fact, only six cases out of 120 breast cancers (5%) were triple negative, while worldwide triple negative breast cancers (TNBCs) account for 15–25% of all breast cancers [24].

Mutation analysis by allele-specific multiplex PCR

In order to rapidly screen for the presence of *BRCA1* 5382insC mutation in a large group of patients, we used an allele-specific multiplex-PCR method [19], adapted previously in our laboratory [20]. We started with exactly the same conditions as used in the adapted method [20], which mean a 50 µL PCR volume containing 100 ng genomic DNA, as presented above. Figure 5 (a and b) present the electrophoretic profiles of the three-primer amplifications of a positive control DNA and of five patients DNA, either after 20 minutes of migration at 5 V/cm (Figure 5a), or after 40 minutes of migration (Figure 5b). We can remark no contamination in the no template control lane, a good separation of two bands indicating the presence of the mutation for the positive control, as well as a single wild-type corresponding band for all investigated patients, which shows their wild-type status for *BRCA1* 5382insC. A good resolution allows the clear discrimination of mutation carriers by this robust and reproducible technique.

We further thought about reducing the PCR reaction volume from 50 to 20 µL. In fact, the technique showed to be robust enough and had good resolution allowing the clear discrimination of mutation carriers, but we also want it to be cheap and rapid, as a pre-screening method adapted to large groups of patients. Therefore, dividing the reaction volume by 2.5 should also permit saving reagents, consumables and time. We kept the same reagent relative concentrations as for the 50 µL reaction, and used 50 ng genomic DNA template instead of 100. The novel conditions allowed saving Taq polymerase, only 0.5 units being used by reaction instead of 1.25. Figure 6 (a and b) present the electrophoretic profiles of the amplifications of a positive control DNA and of five patients DNA, either after 20 minutes of migration at 5 V/cm (Figure 6a), or after 40 minutes of migration (Figure 6b). No contamination was observed in the no template control lane. Otherwise, the novel conditions offered a good separation of two bands indicating the presence of the mutation for the positive control, as well as a single wild-type corresponding band for all investigated patients, which shows their wild-type status for *BRCA1* 5382insC.

As the method showed to be robust and reproducible enough, we applied it for all 170 patients, using simultaneous 95-well plates amplifications and 72-lanes electrophoretic migrations. An example of such work is presented in Figure 7. Even if for a few patients the amplifications did not work (lanes 10–11, up and lanes 13–14, middle), we subsequently re-amplified those DNA and genotyped the corresponding patients.

After genotyping, all analyzed patients (120 breast cancer cases, 50 ovarian cancer cases) did not show to be carriers of the *BRCA1* 5382insC mutation and, consequently, were diagnosed as wild type for the named mutation.

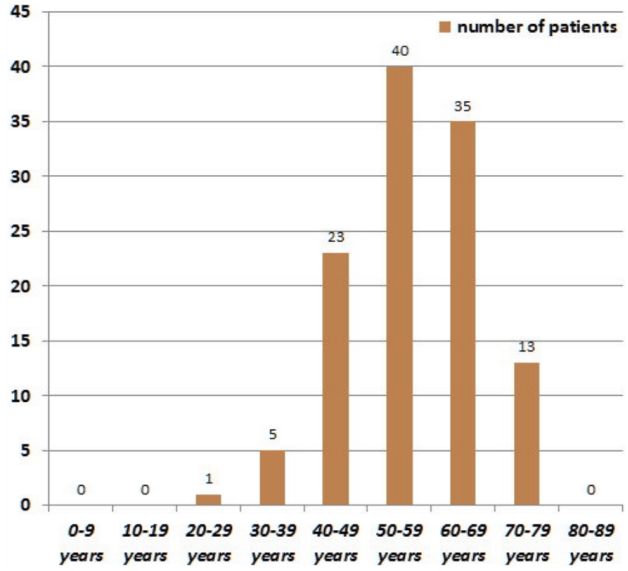

Figure 1 – ***Distribution of breast cancer patients according to the age at diagnosis.***

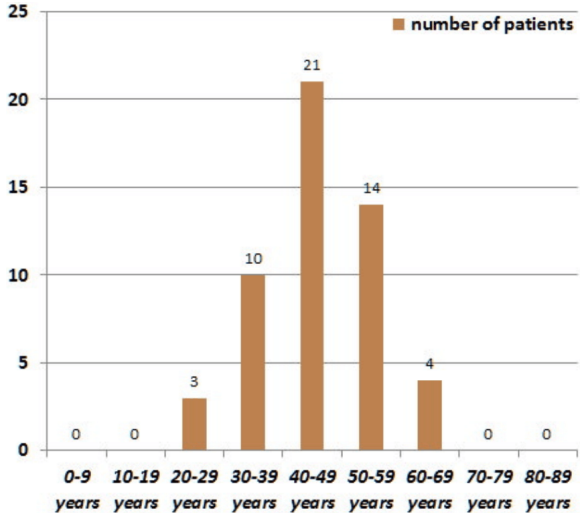

Figure 2 – ***Distribution of ovarian cancer patients according to the age at diagnosis.***

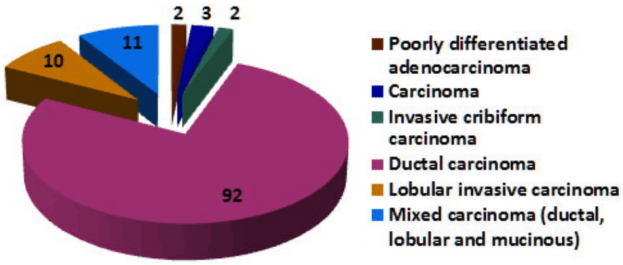

Figure 3 – ***Distribution of breast cancer patients according to the histopathological type.***

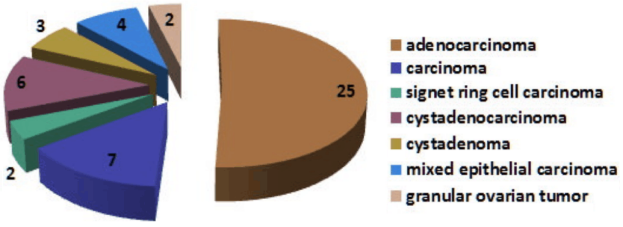

Figure 4 – ***Distribution of ovarian cancer patients according to the histopathological type.***

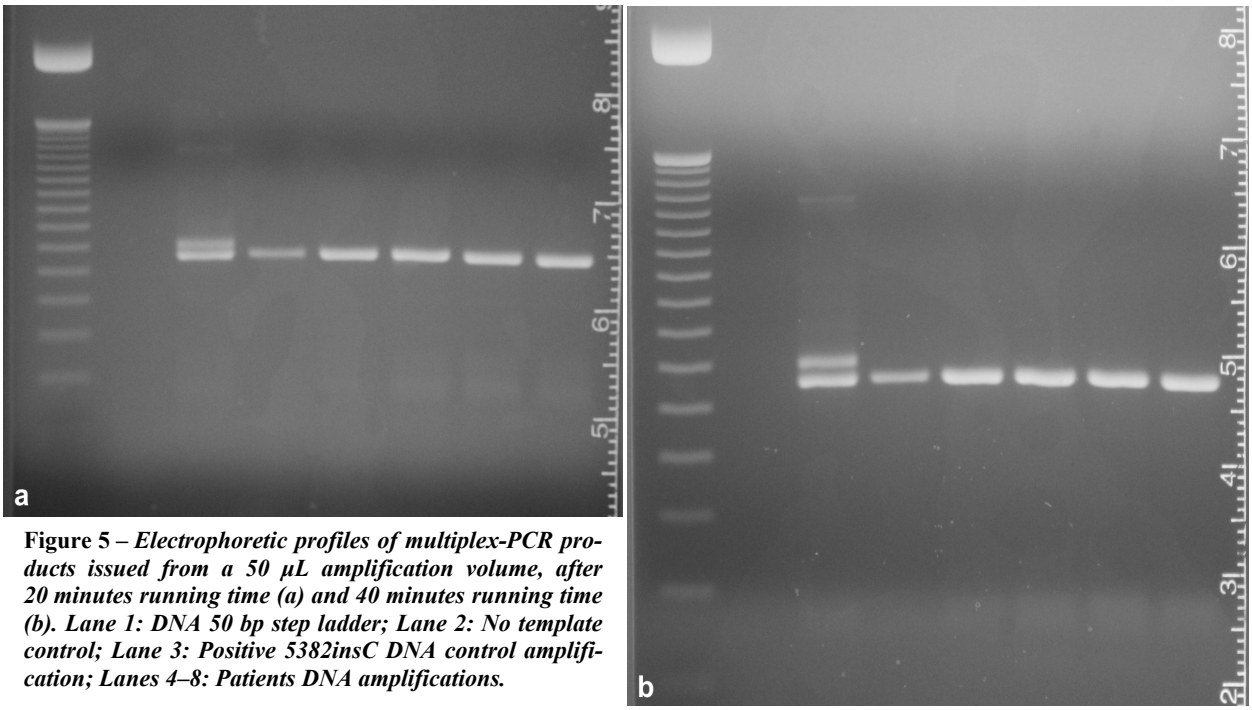

Figure 5 – ***Electrophoretic profiles of multiplex-PCR products issued from a 50 µL amplification volume, after 20 minutes running time (a) and 40 minutes running time (b). Lane 1: DNA 50 bp step ladder; Lane 2: No template control; Lane 3: Positive 5382insC DNA control amplification; Lanes 4–8: Patients DNA amplifications.***

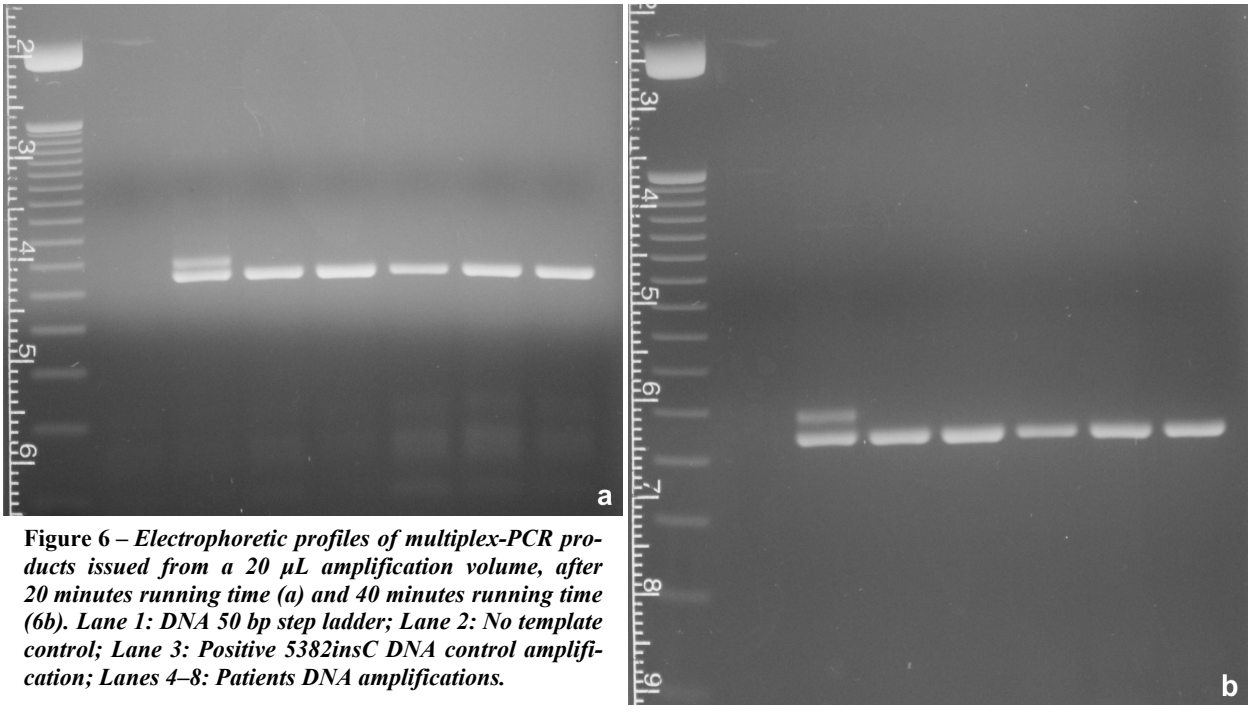

Figure 6 – ***Electrophoretic profiles of multiplex-PCR products issued from a 20 µL amplification volume, after 20 minutes running time (a) and 40 minutes running time (6b). Lane 1: DNA 50 bp step ladder; Lane 2: No template control; Lane 3: Positive 5382insC DNA control amplification; Lanes 4–8: Patients DNA amplifications.***

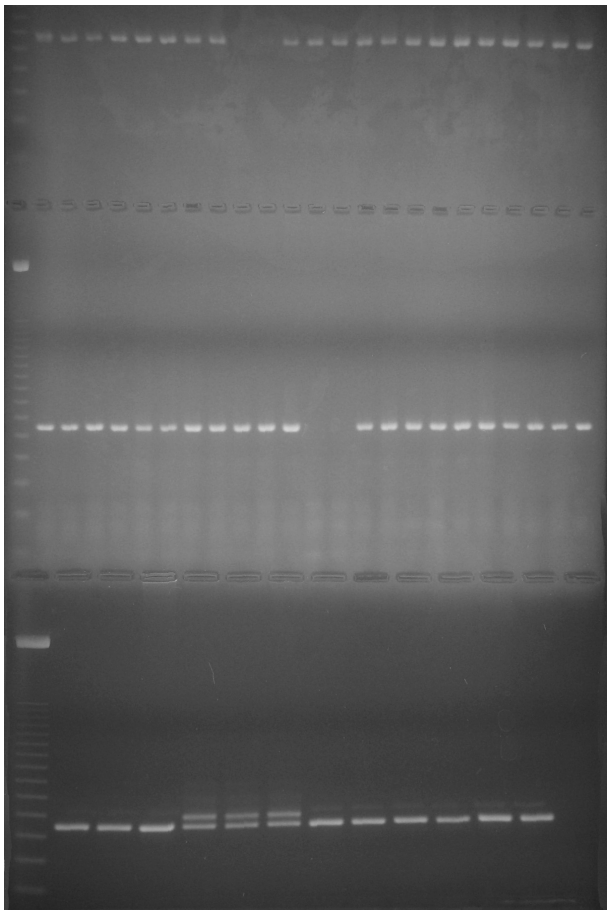

Figure 7 – ***Electrophoretic profiles of multiplex-PCR products issued from 55 patients. Lane 1 (up, middle and down): DNA 50 bp step ladder; Lanes 5–7 (down): Positive control DNA; Lane 14 (down): No template control; All other lanes: Patients DNA.***

Discussion

In the western world, there is strong evidence of decrease in cancer mortality by age tranches after 1990 [5], especially due to development of oncogenetic diagnosis and follow-up. Oncogenetic medical practice mainly concern breast, ovarian and colorectal cancers, and is targeting the hereditary risk factor, the only one that shows positive predictive value justifying the molecular diagnosis. In addition to family aggregation, germ-line predisposition mutations are also responsible for early on-set breast (17–40 years) or ovarian (25–50 years) cancer cases, as well as for cases where multiple breast and other types of cancer (prostate, colorectal, stomach, etc.) are present [25]. Gene expression profiles of breast cancer have defined specific molecular subtypes with clinical, biological and therapeutic implications [26]. Based on these data, breast cancer can be divided into two major categories, estrogen receptor-positive (ER+), and negative (ER-), each consisting of many subgroups. Triple negative breast cancers (TNBCs) represent approximately 15% of breast cancers and are characterized by the absence of expression of estrogen and progesterone receptors (ER/PR) and absence of overexpression of HER2 oncoprotein. Most TNBCs present distinct clinicopathological characteristics and usually appear in women of young age (<45 years). Most often, they are of high histological grade while they represent almost the exclusive phenotype in patients-carriers of *BRCA1* gene mutations [27, 28].

Due to the big size of both *BRCA* genes analyzed for HBOC cases, as well as to the uniform distribution of mutations along the genes, the complete Sanger sequencing, which is the only accepted method in diagnosis, imply huge costs and time. Therefore, a relevant image of the mutation profile for each population is particularly useful for adapting screening and pre-screening strategies. The gold standard technique in molecular diagnostic by genotyping is Sanger dideoxy sequencing of both DNA strands in exons and exon-intro boundaries of predisposition genes (*BRCA1* and *BRCA2* are completely sequenced for HBOC cases). Emerging next generation sequencing technologies are mostly limited to research purposes, although some evidence could open the way, in the future, for diagnosis applications [29]. Full gene sequencing is nevertheless expensive and time consuming, a complete *BRCA* diagnostic being evaluate for Romania at 3000 euros for the duration of one year. Both *BRCA1* and *BRCA2* genes are extremely long and composed of many exons (24 for *BRCA1*, 27 for *BRCA2*) sparing hundreds of genomic DNA kilobases. Moreover, neither mutation hotspot nor neutral genic regions had ever been detected for those genes, so full sequencing is absolutely necessary. The only aspect, which could limit sequencing costs, is the non-uniform distribution of mutation in different populations, with founder or recurrent mutations opening the way for pre-screening methodology.

Since the frequency of *BRCA* mutations in general population is low (prevalence of *BRCA1*/2 mutation carriers is estimated at 0.2% (1/500) [30]), a general screening for mutation carriers in general population is not possible neither suitable. Unfortunately, the majority of models used for probability calculations often underestimate the probability of finding a mutation. Moreover, familial history is also absent or unknown in at least half of all mutation positive families [31] and mutation detection methods vary between most centers. One possible strategy of more rapidly and efficiently detecting *BRCA* mutations is a pre-screening of the most common or recurrent mutations in middle- or big-size populations groups.

In certain countries and ethnic communities, especially in geographically, culturally or religiously isolated populations the *BRCA1/2* mutation spectrum is limited to a few founder mutations [32], while in outbred populations, especially in the western world, mutation spectrum is significantly large. This leads to distinct mutation detection approach strategies applying for molecular diagnosis. Full-gene sequencing is required in oncogenetic diagnosis in the majority of western countries, while adapted pre-screening approach may be useful for founder/recurrent mutations, or even to identify anomalous amplicons prior to sequence. The need of pre-screening techniques is justified by the costs and time-consuming of full sequencing. However, a good knowledge of the target population is essential, comprising mutation diversity and frequency, founder and recurrent effect, geographic and ethnical distribution of haplotypes, briefly a large understanding of population oncogenetics.

Previous research on Romanian population [20, 33] highlighted about 50% of novel/familial mutations, but also 50% recurrent ones, no founder effect having been done yet as a little number of HBOC families already analyzed. Some of the recurrent *BRCA* mutations in our population proved to be common with neighboring countries, while some founder eastern mutations did not appear at all in our population. In the specific case of *BRCA1* 5382insC, the average risk by the age of 70 years is 67% for breast cancer and 30% for ovarian cancer [22]. The 5382insC (c.5266dupC) mutation in *BRCA1* exon 20 is the second most frequently reported mutation in the BIC database, being very prevalent in Central and Eastern Europe. This mutation is found in a various frequency in HBOC families from Poland, Russia, Belarus, Hungary, Slovenia, Latvia, Lithuania or Czech Republic [17]. Our previous results [20] highlighted the importance of rapid and cheap allele-specific multiplex-PCR approach for 5382insC detection. Subsequent work [33] revealed the presence of 5382insC in two different HBOC families, with distinct cancer phenotypes, out of a group of 20 families, which strongly suggested a recurrent effect of the mutation in our population.

In the present work, we screened for *BRCA1* 5382insC mutation in consecutive series of breast and ovarian cancer patients, independently of their family cancer history. Surprisingly, not one carrier of the mutation was identified out of 120 breast and 50 ovarian cancer patients. The results somehow interfere with previous works and seriously decrease the probability for 5382insC to be the most important recurrent mutation in Romania. This directly influence the pre-screening strategy for mutations detection, proving the higher importance of complete sequencing screening, more than in neighboring populations anyway.

Conclusions

No presence of the *BRCA1* 5382insC mutation was observed in consecutive groups of 120 breast and 50 ovarian cancer patients. This result is decreasing the importance effect previously thought for 5382insC in our population. The lesser recurrent effect also imply an adaptation of mutation detection strategies, targeting the methodology more alike to western oncogenetic centers, with strategies more focused on complete gene sequencing rather than investing in pre-screening rapid techniques.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

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