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Original paper

BRCA1 AND *BRCA2* GENES MUTATION ANALYSIS IN PATIENTS WITH A FAMILY HISTORY OF BREAST AND OVARIAN CANCER

Irene Konstantopoulou[1], Radmila Janković[2], Lidija Raičević[2], Angela Ladopoulou[1], Sophia Armaou[1], George Nikolopoulos[1], Nikos Pandis[3], George Nasioulas[4], Siniša Radulović[2], Drakoulis Yannoukakos[1]

[1]Molecular Diagnostics Lab., IRRP, »Demokritos« National Centre of Scientific Research, Aghia Paraskevi, 153 10 Athens, Greece
[2]Institute of Oncology and Radiology of Serbia, Belgrade, Serbia & Montenegro
[3]Department of Genetics, Saint Savvas Anticancer Hospital, Athens, Greece
[4]HYGEIA Molecular Biology Dept, Diagnostic & Therapeutic Center of Athens, Athens, Greece

Summary: Hereditary breast and ovarian cancer syndromes can be caused by loss-of-function germline mutations in one of the tumour suppressor genes *BRCA1* and *BRCA2*. In order to characterize these mutations in the Greek population we have been collecting samples from breast/ovarian cancer patients with a family history in collaboration with a large number of Greek Hospitals. Our DNA bank contains samples from more than 300 patients, corresponding to approximately 250 families. In terms of family history this group consists of three subgroups: (i) very early onset (<30 yrs) without family history (10%); (ii) moderate family history (2 members affected, < 50 yrs) (40 %) (iii) strong family history (3–7 members affected) (50 %). Screening of *BRCA1* and *BRCA2* genes in 150 patients has revealed deleterious mutations in 39 unrelated patients. 5382insC has been found in 11 unrelated families. In summary, the mutation spectrum of *BRCA1* and *BRCA2* genes in the Greek population seems to be composed by an elevated frequency of 5382insC with the rest being novel or recurrent mutations with low frequency in both genes. Screening of *BRCA1* gene is also in progress in collaboration with the Institute of Oncology and Radiology of Serbia. DNA samples have been collected from 32 Serbian families with a family history of breast ovarian cancer. Direct sequencing in exons 2, 5 and 20 of all these samples revealed only one deleterious mutation (C61G) in exon 5 of *BRCA1* gene. Screening of the remaining exons is in progress.

Key words: BRCA1, *BRCA2*, breast ovarian cancer, familial, Serbia, Greece

Introduction

Hereditary breast and ovarian cancer syndromes can be caused by loss-of-function germline mutations in one of the tumour suppressor genes *BRCA1* and *BRCA2* (1–3). The lifetime risk for carriers of germline mutations in these genes is estimated to be as high as 50 to 85 percent for breast cancer and 15 to 45 percent for ovarian cancer. Although *BRCA1* and *BRCA2* mutations account for a substantial proportion of inherited breast and ovarian cancer (4), it seems likely that additional susceptibility genes will be discovered in the future (5). Approximately 10% of ovarian and 7% of breast cancer cases in the general population are estimated to be carriers of a breast/ovarian cancer susceptibility gene; these women are found primarily in families characterized by multiple cases of early onset of breast cancer (6).

The frequency of *BRCA1* and *BRCA2* mutation carriers in women with breast or ovarian cancer (or both) depends on the study population (4, 7). More than 500 different mutations have been found in the above genes; moreover, different mutations have been observed in different geographical regions (*Figure 1*). While the range of pathological alterations in some countries or ethnic groups is mainly limited to a few (3 mutations for 90% of Ashkenazim, 3 mutations for 50% of Norwegians) or even one (Iceland) mutations, in other countries the broad mutational spectrum poses difficulties in detection efforts and high-risk population management (4, 7, 8). Therefore, the analysis in each population group is necessary.

Address for correspondence:
yannouka@rrp.demokritos.gr

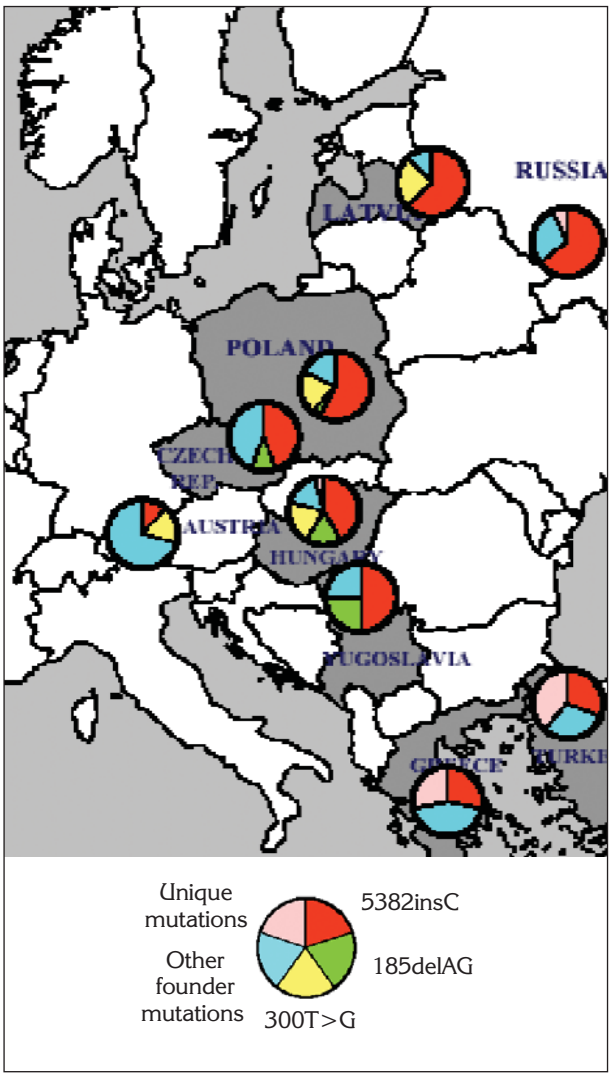

Figure 1. *BRCA1* gene mutation spectrum in Central-Eastern European countries (From Professor Edith Olah – European Association for Cancer Research, www.internationales-buero.de/veranstaltungen/528/02-A12_Olah.pdf)

The most common disease-predisposing allele in Russia, 5382insC, is also the most common among Europeans as a whole and has migrated far from the Baltic area where it probably originated (4). This mutation has been the only one found in a relatively high frequency in southern countries neighbouring to Greece, such as Italy (9), Yugoslavia (10), and Turkey (11) (See also (12)).

Breast cancer is among the most common malignancies in Greek women (13). In our effort to determine the contribution of *BRCA1* and *BRCA2* deleterious mutations to the development of breast and/or ovarian cancer in the Greek population, we constructed genomic DNA and data banks from families with many cases of breast/ovarian cancer. The methodology employed involves mainly techniques such as PCR, dHPLC, PTT, and automated DNA sequencing. The same task was undertaken by the Institute of Oncology and Radiology of Serbia and DNA samples have been collected from Serbian families.

We have screened all exons and intron-exon boundaries of *BRCA1* and exons 10 and 11 of *BRCA2* gene in 200 Greek patients with breast/ovarian family history. From the 32 Serbian DNA samples collected, *BRCA1* exons 2, 5 and 20 have been directly sequenced so far.

Materials and Methods

Patients and their families – The Greek genomic DNA bank consists of approx. 300 samples from breast and/or ovarian cancer patients with family history. Inclusion criteria are having at least one first- or second-degree relative with breast cancer under age 50 or ovarian cancer at any age. Families were selected under informed consent from patients attending the participating hospitals, in collaboration with the Hellenic Cooperative Oncology Group (HECOG). The Serbian genomic DNA bank was constructed at the Institute of Oncology & Radiology of Serbia.

Mutation Screening – All nucleotide numbers refer to the wild-type cDNA sequences of *BRCA1* and *BRCA2* as reported in GenBank (accession numbers U14680 and U43746.1, respectively). Primer pairs were used to amplify all exons and intron-exon boundaries from genomic DNA extracted from patient samples with routine techniques. Primer selection was made from BIC database at http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/Member/brca1_mutation_database.html. Genomic DNA was amplified by the Polymerase Chain Reaction (PCR) in a Perkin-Elmer 2400 Thermocycler (Perkin Elmer, CA, USA).

PTT Analysis – Exon 11 of *BRCA1* and exons 10 and 11 of *BRCA2* were amplified in the presence of forward primers containing a T7 promoter and transcription/translation initiation sequence as described (Hogervorst et al., 1995). The PCR products were then subjected to an *in vitro* transcription/translation initiation reaction in a reticulocyte lysate system (Promega), electrophoresed at a 12% sodium dodecyl sulfate polyacrylamide gel, fixed, dried and autoradiographed.

DNA Sequencing – PCR products were sequenced directly with the same forward and, when needed, reverse primers used for PCR amplification. Sequencing was done using an ABI PRISM DyeDeoxy Terminator Cycle Sequencing Kit and an ABI 310 Genetic Analyzer (Perkin Elmer, Applied Biosystems, CA, USA), according to manufacturer's instructions. Any mutation found was confirmed on a second DNA sample isolated from a duplicate tube of blood followed by sequencing in both forward and reverse directions.

Results

In order to characterize these mutations in the Greek population we have been collecting samples from breast/ovarian cancer patients with a family history in collaboration with a large number of Greek Hospitals. Our DNA bank contains samples from more than 300 patients, corresponding to approximately 250 families. In terms of family history this group consists of three subgroups: (i) very early onset (<30yrs) without family history (10%); (ii) moderate family history (2 members affected, <50yrs) (40%); (iii) strong family history (3–7 members affected) (50%).

BRCA1 mutation analysis in Greek patients

Screening of BRCA1 exon 11 by PTT revealed six truncated protein products corresponding to different frameshift mutations. In a family harbouring the 1623del5 exon 11 mutation (#98), a healthy carrier was found [(12); data in preparation].

Two deleterious mutations were identified by direct sequencing in exon 20, 5382insC and R1751X. The known founder mutation 5382insC was found in eleven unrelated families; six of these had a very strong family history with at least four members affected. In three of those families (#177, 180, 198) where blood samples were available from an affected relative, the presence of 5382insC was documented in all of them. In one family (#180) a healthy carrier was also identified (see *Figure 2* for a typical 5382insC family pedigree). The nonsense mutation R1751X was observed in three cases, a patient (#175) with bilateral breast cancer, but without any family history, and in two more patients (#189, 290) who developed breast and ovarian cancer [(12,14); and data in preparation].

The splice-junction mutation 5586G>A, corresponding to an alteration of the last base in exon 23, was identified by direct sequencing in two unrelated patients. After analysis at the RNA level, it was confirmed that this mutation results in an alternatively spliced form of *BRCA1* and therefore is disease-associated (15).

A rare missense mutation, G1738R, was identified in exon 20 of *BRCA1* in nine unrelated patients. This mutation is reported only twice in the BIC database by Myriad Genetics. It has been shown that a different alteration in the same residue, G1738E, results in loss of *BRCA1* protein function (18, 19); this residue is located inside the linker of the two BRCT domains in the C-terminal region of *BRCA1* (20). Our preliminary functional and segregation analysis data suggest that this mutation may be a deleterious one; in addition it may be a founder mutation for the Greek population (data in preparation).

Some other rare missense mutations were identified only once inside the BRCT domains of *BRCA1*, including A1823T (exon 23), V1833M (exon 24), P1856S (exon 24), which need further studies in order to classify them (14).

BRCA2 mutation analysis in Greek patients

Preliminary search for mutations on the *BRCA2* gene was performed by using the PTT analysis to exons 10 and 11. PTT analysis led to the identification of 2 truncated protein products, in families #144 and #85 respectively. Sequence analysis of the regions likely to contain the protein-terminating alterations revealed two frameshift mutations. More mutations were found using SSCP and subsequent DNA sequencing in patients AB15, H78, H41, H43, H44, and H46. In all, six different *BRCA2* frameshift mutations were identified in eight unrelated families, of which three are novel mutations (14, 19) (*Table I, Figure 3*).

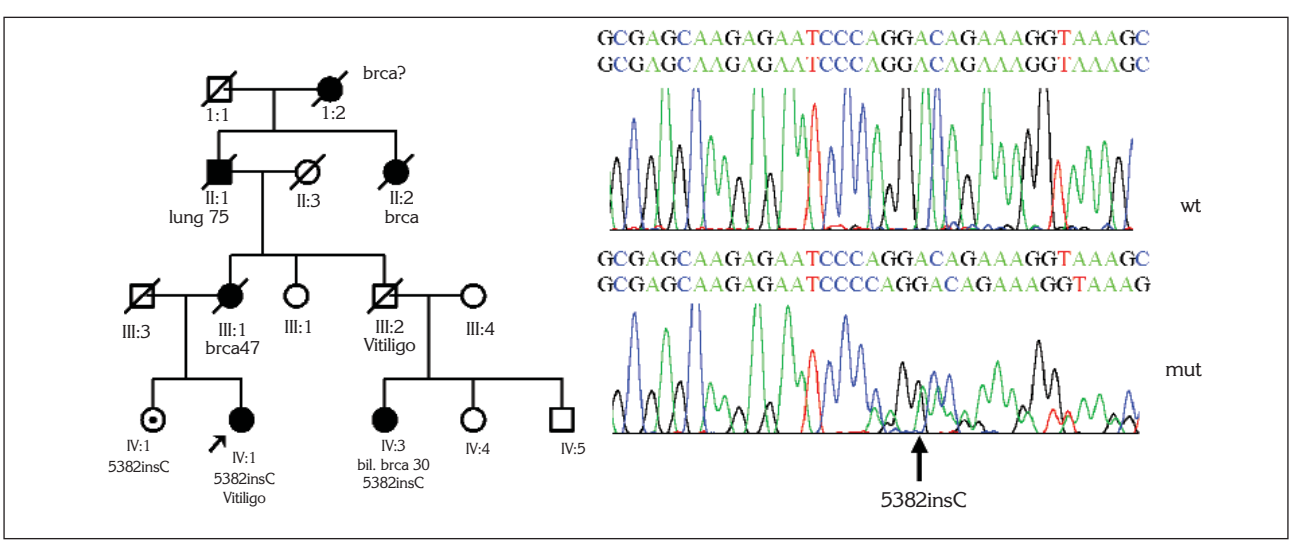

Figure 2. Typical pedigree from a family with history of breast cancer carriers of the most common mutation found in the Greek population (5382insC), and DNA sequence analysis of *BRCA1* exon 20. wt: wild type, mut: mutant

Table I Phenotypes and mutations identified in Greek breast/ovarian cancer families (Smaller patient number in each family denotes family number, G1738R families are not included, see text).

Patient No.	No. of BrCa cases (age of onset)	No. of OvCa cases (age of onset)	Gene / exon	Mutation	Effect	Comments / other cancers
98, 314, 315	1(25)	1(44)	*BRCA1* / *exon11*	1623del5	ter505	One more case of endometrial ca
99	1 (xx)	4 (33,40,40, xx)	*BRCA1* / *exon11*	3099delT	ter999	Br & OvCa in the same patient
145	1 (62)	3 (40, 72, xx)	*BRCA1* / *exon11*	3277insG	ter1059	Br & OvCa in the same patient
230	1 (40)		*BRCA1* / *exon11*	R1203X	Arg to stop	Proband adopted (unknown family history)
64	2 (31, 35)	1 (48)	*BRCA1* / *exon11*	3741insA	ter1218	One bilateral BrCa
211	1 (48)		*BRCA1* / *exon11*	3896delT	ter1263	Br & OvCa in the same patient
97	2 (35, 42)		*BRCA1* / *exon20*	5382insC	ter 1829	
113, 236	4 (39, xx, xx, xx)	4 (75, 74, xx, xx)	*BRCA1* / *exon20*	5382insC	ter 1829	Br & colon ca in the same patient
172	3 (59, 39, 30)		*BRCA1* / *exon20*	5382insC	ter 1829	One bilateral BrCa
177,187	5 (41, 36, 52, xx, xx)		*BRCA1* / *exon20*	5382insC	ter 1829	
180, 181	4 (47, 47, 30, xx)		*BRCA1* / *exon20*	5382insC	ter 1829	
198, 199, 200	4 (35, 50, 50, xx)	4 (45, 60, 53, 80)	*BRCA1* / *exon20*	5382insC	ter 1829	Vitiligo
280		1 (56)	*BRCA1* / *exon20*	5382insC	ter 1829	
296	1 (48)		*BRCA1* / *exon20*	5382insC	ter 1829	Br & OvCa in the same patient
AB23	2 (47, 47)		*BRCA1* / *exon20*	5382insC	ter 1829	
AB21	2 (47, 47)		*BRCA1* / *exon20*	5382insC	ter 1829	
AB07	3 (39, 45, 51)		*BRCA1* / *exon20*	5382insC	ter 1829	
175	1 (45)	1 (42)	*BRCA1* / *exon20*	***R1751X***	Arg to stop	Bilateral BrCa
189	2 (41, 32)	1 (xx)	*BRCA1* / *exon20*	***R1751X***	Arg to stop	Br & OvCa in the same patient
290	1 (xx)	1	*BRCA1* / *exon20*	***R1751X***	Arg to stop	Br & OvCa in the same patient
155	2 (40, 50)		*BRCA1* / *exon23*	*5586G>A*	Splice site	Br & OvCa in the same patient
178	4		*BRCA1* / *exon23*	*5586G>A*	Splice site	Leukaemia, testicular, gastric, head & neck ca
144	4 (66, 33, 34, 39)		*BRCA2* / *exon10*	2024del5	ter 599	One case of male BrCa and one of prostate ca
H78	2 (67, xx)		*BRCA2* / *exon10*	2024del5	ter 599	
		1 (xx)				
AB15	2 (32, 48)		*BRCA2* / *exon11*	3034del4	ter 958	One case of prostate cancer
H43	4 (62, xx, xx, xx)		*BRCA2* / *exon11*	3058delA	ter 959	Bilateral BrCa
H41	2 (59, xx)		*BRCA2* / *exon11*	4047delG	ter 1334	Br & OvCa in the same patient
H46	4 (31, xx, xx, xx)		*BRCA2* / *exon11*	4047delG	ter 1334	
H44	3 (32, xx, xx)		*BRCA2* / *exon11*	6024delTA	ter 1943	
85	3 (36, 38, xx)		*BRCA2* / *exon11*	6631del5	ter 2137	

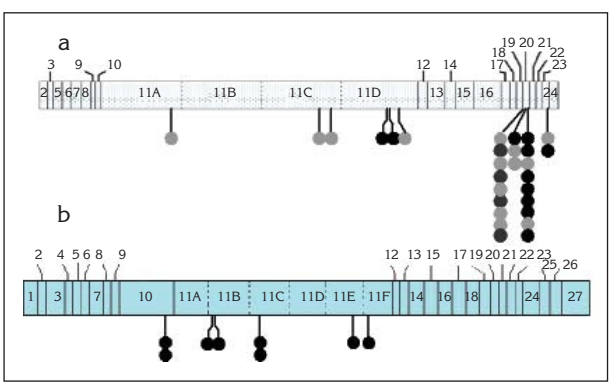

Figure 3. Mutational spectrum of *BRCA1* (a) and *BRCA2* (b) genes found in Greek families with history of breast ovarian cancer. Every cycle represents one family. Black cycles: families with breast cancer cases only; grey cycles: breast and ovarian cancer cases.

BRCA1 mutation analysis in Serbian patients

The same study has been undertaken in collaboration with the Institute of Oncology and Radiology of Serbia and DNA samples have been collected from 32 Serbian families. Direct sequencing in exons 2, 5 and 20 of all these samples revealed only one deleterious mutation, C61G in exon 5 of the *BRCA1* gene. This study is in progress.

Discussion

Our ongoing study of the *BRCA1* and *BRCA2* mutational spectrum in the Greek population reveals that it is comprised mainly by mutations in exons 11 and 20 of *BRCA1* and 10 and 11 of *BRCA2*. Of 200 breast/ ovarian cancer patients from medium to high-risk families, 39 carry loss-of-function mutations (19.5%) [(12,14,15,19); data in preparation].

Eleven out of 39 families with a deleterious *BRCA1* or *BRCA2* mutation carry the 5382insC mutation. Therefore the founder mutation 5382insC is approximately 28% of the carrier cases identified. It has to be highlighted at this point that our study group contains patients from families with no (<35 years old), medium (2 members affected) and strong (three or more members affected) family history, while families carriers of 5382insC usually have a strong family history. In a multinational study, the single Greek patient reported was a carrier of 5382insC (20). This is not surprising as this mutation has been observed in high frequency by us, as well as in very high frequencies in North Eastern European countries (mainly Hungary and Russia) with geographic and historical proximity to Greece (4, 7). In all, 5382insC exhibits a frequency of 5.5% among families in our study group.

Several missense unclassified variants have also been characterized. Among them, mutation G1738R presents a particular interest, as it is found in eleven unrelated families (5.5% in breast/ovarian cancer families and 28% among carriers) and seems to be restricted in the Greek population. This mutation is reported only twice in BIC by Myriad Genetics except the entries from our group. Recently it was also observed in a patient of Greek origin in Peter MacCallum hospital, Melbourne, Australia (Dr. Alasdair Hunter, personal communication). We are proposing that this mutation may be a deleterious one because (i) it never co-occurs with a truncating mutation, (ii) replacement of the same Gly1738 residue by Glu was shown to cause loss of function *in vitro* (16), (iii) Gly1738 is conserved among all BRCA1s and is structurally conserved in 53BP1 proteins that also contain a tandem of BRCT repeats, (iv) in the crystal structures of human BRCA1 (21) and of human 53BP1 (22), Gly1738 and its equivalent Gly1851 are located at the beginning of the flexible linker connecting the two BRCT repeats, which in addition participates in the BRCT inter-repeat interface formation, and (v) it shows co-segregation with the disease in two breast/ovarian cancer families (total 4 patients and 1 healthy carrier).

Five novel mutations were identified in the Greek families studied, two in *BRCA1* (3099delT and 3277 insG, both in exon 11) and three in *BRCA2* (3058delA, 4147delG, and 6024delTA, all in exon 11).

It is worth mentioning that *BRCA1* exons 2 and 5, where common mutations are described in other populations (185delAG and C61G), were sequenced directly in over 150 patients but no sequence variation was observed, contrary to other highly variable exons as 11, 16, 20, etc.

Our results indicate that *BRCA1* and *BRCA2* are responsible for a significant part of hereditary site-specific breast cancer cases in Greece. The mutation spectrum of the Greek population seems to be composed by an elevated frequency of 5382insC, the rest being novel or recurrent mutations with low frequency in both genes. Although 5382insC is the most frequent deleterious mutation observed in the Greek population so far (until G1738R is proven to be deleterious beyond any remaining doubt), mutation detection strategies should optimally include complete analysis of *BRCA1* and *BRCA2* genes because many other unique or low frequency loss-of-function mutations exist in this population. Screening of a larger patient group remains also necessary.

Table II Frequencies of deleterious* *BRCA1/2* mutations found in the Greek population

Gene	Exon	Exon or Specific mutation	% of mutation carriers (n=39)	% of families studied (n=200)
BRCA1	exon 20	5382insC	28	5.5
		G1738R	28	5.5
		R1751X	7.7	1.5
		exon 20 TOTAL	**63.7**	**12.5**
	exon 23	5586G>A	5.1	1.0
	exon 11	exon 11 TOTAL	15.4	3.0
BRCA2	exon 10	2024del5	5.1	1.0
	exon 11	exon 11 TOTAL	15.4	3.0

* G1738R is included (see text)

For the time being, we propose the following strategy for the screening of these large genes, as indicated by the mutation frequencies observed in our study population, summarized in *Table II*: A total frequency of 64% among carriers for BRCA1 exon 20 mutations denotes as obligatory that screening of this exon should be the first thing to do. Subsequent steps should include screening of exons 11 and 23 of *BRCA1* and/or 11 and 10 of *BRCA2*; consideration of several prognostic factors (e.g. cases of ovarian cancer, male breast cancer, early onset breast cancer, oestrogen receptor status) would facilitate the choice of priority given to *BRCA1* or *BRCA2*.

Taking the previous experience into account, the research collaboration between the Serbian and Greek groups is aimed at the completion of the analysis of *BRCA1* and *BRCA2* mutational spectrum in the two examined populations. Analysis of Serbian families is in progress. This knowledge will contribute significantly to the development of valid diagnostic methods at a minimum cost.

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ANALIZA MUTACIJA U *BRCA1* I *BRCA2* GENIMA KOD PACIJENATA SA FAMILIJARNOM ISTORIJOM KANCERA DOJKE I OVARIJUMA

Irene Konstantopoulou[1], Radmila Janković[2], Lidija Raičević[2], Angela Ladopoulou[1], Sophia Armaou[1], George Nikolopoulos[1], Nikos Pandis[3], George Nasioulas[4], Siniša Radulović[2], Drakoulis Yannoukakos[1]

[1]Molecular Diagnostics Lab., IRRP, »Demokritos« National Centre of Scientific Research, Aghia Paraskevi, 153 10 Athens, Greece
[2]Institute of Oncology and Radiology of Serbia, Belgrade, Serbia & Montenegro
[3]Department of Genetics, Saint Savvas Anticancer Hospital, Athens, Greece
[4]HYGEIA Molecular Biology Dept, Diagnostic & Therapeutic Center of Athens, Athens, Greece

Kratki sadržaj: Nasledni kanceri dojke i ovarijuma mogu biti uzrokovani germinativnim mutacijama u tumor supresornim genima BRCA1 i BRCA2. U nastojanju da ove mutacije karakterišemo u grčkoj populaciji, u saradnji sa velikim brojem grčkih bolnica, sakupljali smo uzorke pacijenata sa kancerom dojke/ovarijuma koji su imali familijarnu istoriju bolesti. Naša DNK banka sadrži uzorke više od 300 pacijenata, članova oko 250 familija. U odnosu na familijarnu istoriju bolesti, ova grupa pacijenata obuhvata tri podgrupe: (i) pacijente sa vrlo ranom pojavom bolesti (<30 godina), bez familijarne istorije (10%); (ii) pacijente sa srednje izraženom familijarnom istorijom (2 člana porodice pogođena bolešću, <50 godina) (40%); (iii) pacijente sa jako izraženom familijarnom istorijom pojave bolesti (3–7 članova pogođenih bolešću) (50%). Analizom BRCA1 i BRCA2 gena kod 150 pacijenata, detektovane su delecije kod 39 pacijenata koji nisu bili u srodstvu. 5382insC mutacija locirana je kod 11 familija. Zaključak je da mutacioni spektar BRCA1 i BRCA2 gena u grčkoj populaciji podrazumeva povećanu zastupljenost mutacije 5382insC, dok ostatak čine nove ili već opisane mutacije ali sa malom zastupljenošću u oba gena. U okviru saradnje sa Institutom za onkologiju i radiologiju Srbije, u toku je analiza BRCA1 gena u uzorcima DNK prikupljenih kod pacijenata iz 32 srpske familije sa familijarnom istorijom kancera dojke i ovarijuma. Direktnim sekvenciranjem eksona 2, 5 i 20 u svim ovim uzorcima, detektovana je samo jedna delecija (C61G) u eksonu 5 BRCA1 gena. Analiza preostalih eksona je u toku.

Ključne reči: BRCA1, *BRCA2*, kancer dojke/ovarijuma, familijarni, Srbija, Grčka

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