The novel exon 11 mutation of BRCA1 gene in a high-risk family*

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Germline mutations in the BRCA1 and BRCA2 genes are required for the initiation of the development of hereditary forms of breast and ovarian cancer, which represent 10–15% of all cases. The course of the disease varies from case to case that can be due even to the possibility of multiple genetic changes including inactivation of other tumor suppressor genes – TP53 and APC genes or activation of oncogenes, especially K-ras oncogene. The combination of these changes results in an early expression of the broad variety of malignancies.

The analyzed proband (II-5) comes from a high-risk family, in which various types of cancer were observed. The novel BRCA1 mutation in exon 11 (2057delCAGTGAAGAG) was detected by SSCP, HDA techniques and confirmed by automatic sequencing. The same deletion was observed in DNA sample of her first daughter (III-1), but DNA of her second one was without any mutational changes (III-2). Due to the occurrence of different types of cancer in this family, the incidental mutations in the APC; resp. TP53 tumor supressor genes and K-ras oncogene were searched as well. Any mutation was found after sequencing of SSCP interesting exons of these genes. The reasons for such strong malignant manifestation in this high risk family are discussed.

Key words: Breast and ovarian cancer, BRCA1 gene germline mutations, single strand conformation polymorphism (SSCP), heteroduplex analysis (HDA), APC gene, TP53 gene, K-ras oncogene.

According to the recent statistical data published by the World Health Organisation, the breast and ovarian cancer are the leading causes of women's mortality between age 40 and 55. All over the world there are registred more than 1 million new cases per year with 30% of mortality [18].

Many studies suggest that epigenetic factors as menarche before 12 years of life, first pregnancy at the age over than 30 or childlessness might increase the risk for breast cancer, anyway family history remains one of the strongest risk factor [3]. Based on genetic observations, at least two genes as candidates of susceptibility have been identified – BRCA1 [10] and BRCA2 [21] localized on 17q21 and 13q12-13, respectively. Recently, another candidate gene BRCAX has been emerged (13q21) [6].

In some families with inherited mutation in one of the mentioned breast and ovarian cancer genes might be frequently expressed even by another types of cancer as well. The BRCA1 mutation carriers have increased risk of colon cancer, whereas male ones carries increased risk of prostate cancer [12].

Some high-risk families are characterized by the presence of germline mutations not just in one disease-responsible gene but in other genes participating in hereditary forms of cancer, too. The example is occurence of both germline mutations in the APC gene responsible for the initiation of hereditary colon carcinoma together with germline mutation in the TP53 gene. The combination of these two germline mutations was expressed in a very early onset of the disease [22]. TP53, APC or mismatch repair genes have been already identified as rare causes of hereditary breast and ovarian cancer [1]. Particularly, inherited mutation in the TP53 gene causes the Li-Fraumeni syn-

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drome which is occasionally characterized by presence of breast cancer [19].

Not only mutations in tumor-supressor genes are responsible for the malignant process but even inactivation of proto-oncogenes are involved in the development of human ovarian cancer. Some ovarian tumors display K-ras oncogenes with 12th or 13th codon mutations. K-ras activation is specific for mucinous tumors including adenomas [4].

In this report we describe a high-risk family with frequent occurence of breast and ovarian cancer as well as colon, oesophagus and lung cancer.

Case report. The heterogenity of different types of cancer is shown on the pedigree of analyzed affected family (Fig. 1). Proband's age (II-5) of diagnosing of bilatheral ovarian cystadenopapilocarcinoma was 50 years. Schedule of consequent therapy included hysterectomy and bilateral ovarectomy, omentectomy, chemotherapy and X ray therapy. Proband was 3 years in remission after this treatment. A polyp on ascending colon was found by preventive colonoscopy, which was histologically characterized as adenocarcinoma. Eight years later 4–5 cm infiltrative ductal carcinoma was diagnosed in the right breast. Patient died two years later due to progression both of ovarian and breast carcinomas. Although her two daughters (III-1; 2) seemed to be clinically healthy, genetically were investigated.

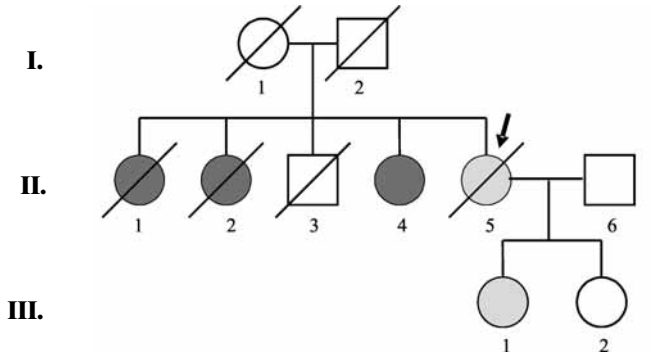

Figure 1. Pedigree of a high risk family suspected for hereditary form of breast and ovarian cancer. Mother (II-5) died of both ovarian and breast carcinomas, her first daughter (III-1) is positive for BRCA1 mutation, her second daughter (III-2) hasn't mentioned mutation. Proband's mother (I-1) died of colon cancer and her father (I-2) of larynx cancer. Proband's sisters (II-1; 2) died of ovarian cancer and the other one (II-4) is after ovarial surgery. Her brother (II-3) died of lung cancer.

Some additional facts from family history: proband's mother died of colon cancer in her 58 years, her father died of oesophagus carcinoma and her two sisters died of ovarian carcinoma (age of onset of both was 39). Her other sister is 60 and she is 10 years after surgery for ovarian tumor. Proband's brother died of lung cancer at the age of 60.

Material and methods

The examined family and DNA isolation. The members of the breast and ovarian cancer high risk family have been collected in collaboration with the National Cancer Institute and tested for BRCA1 gene mutations. Genomic DNA was isolated from peripheral lymphocytes as described by KIRCHHOFF et al [7].

Single Strand Conformation Polymorphism. The PCR for the SSCP have been performed in 25-μl reaction volume including 100–200 ng of genomic DNA, 200 μM dNTP mix, 10 x PCR buffer (10mM Tris-HCl, 50mM KCl, 0.2 mg/ml BSA), 40 pmol of each primer, 0.5U of Taq polymerase (Platinum®Taq DNA Polymerase, GibcoBRL). The PCR cycling regime comprised an initial denaturation step at 94 ˚C for 5–15 min to activate Taq polymerase. The next PCR's steps were subjected to 30 cycles as described: 94 ˚C/1 min, 60 ˚C/1 min, 72 ˚C/1 min. The PCR samples were denatured 5 min at 94 ˚C, placed on ice for 5 min to prevent reannealing, loaded into a 6% polyacrylamide gel and electrophoresed at 4 ˚C for 20 hours at 5W. The gel was silverstained: 15 min fixation in 10% ethanol, 10 min incubation with 1% HNO_3, 30 min incubation with 0.2% $AgNO_3$ containing 1 μl/ml formaldehyde, developing with 3.5% Na_2CO_3 containing 0.5 μl/ml formaldehyde, until the bands appear fixation with 10% acetic acid [11, 17].

Description of the primers used for SSCP. We analyzed 22 exons of BRCA1 gene using primers according to WAGNER [20]. The largest exon 11 (3500bp) is divided into 14 subregions from AB to WX. Subregion 11J is localized between 1817–2100bp and the sequences of the primers used for its' targeting were as follows:

11Jfor 5' CTA AAA AGA ATA GGC TGA GGA GGA AGT
11Jrev 5' CAG CTC TGG GAA AGT ATC GCT G

The primers used for analysis of K-ras exons 1 and 2:
Kras1for 5' CCT GCT GAA AAT GAC TGA ATA
Kras1rev 5' TCT ATT GTT GGA TCA TAT TCG
Kras2for 5' ATT CCT ACA GGA AGC AAG TAG
Kras2rev 5' CTA TAA TGG TGA ATA TCT TCA

The primers used for analysis of TP53 exons 5, 7 and 8:
TP53e5for 5' TAC TCC CCT GCC CTC AAC AAG AT
TP53e5rev 5' ATC GCT ATC TGA GCA GCG CTC AT
TP53e7for 5' GGC TCT GAC TGT ACC ACC ATC
TP53e8rev 5' ACC TCG CTT AGT GCT CCC TG

DNA sequencing. The PCR products have been purified by Exo-SAP-IT kit according to the manufacturer protocol (Amersham Biosciences) to remove the nucleotides and primers. The sequencing reaction was performed using primers for PCR, fluorescent dyes based on ABI Prism Big

Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer Applied Biosystems) and afterward the extension products were purified by AutoSeq G-50 columns (Amersham Biosciences). Purified products were sequenced on an automated capillary DNA sequencer (ABI-PRISM 310 Genetic Analyzer, Applied Biosystems).

The obtained sequences have been analyzed and compared with the non-redundant nucleotide and protein database using the BLASTN and BLASTX programs on the NCBI server.

Results

We have analyzed DNA from three members of a high risk family having occurence not only breast but also broad variety of cancer sites: mother (II-5) who was clinically positive for breast cancer and her two daughters (III-1; 2) without any symptoms of disease. The PCR products of exons 2–10; 12–24 and 14 segments of exon 11(AB-WX) of the BRCA1 gene were prepared according to WAGNER's pattern [17, 20] and screened by SSCP technique. We have revealed extra bands in segment J of exon 11 in samples II-5 and III-1 (Fig. 2, lines 1, 2). Abnormalities in SSCP conformation were confirmed by creation of heteroduplexes using HDA technique of the same segment (Fig. 3, lines 1, 2). Screening of other exons of BRCA1 gene revealed some common polymorphisms which effect in induction of tumor predisposition was not described till now.

Detail analysis of expected mutations in segment 11J of samples II-5, III-1 was performed by automatic sequencing. The novel mutation 2057delCAGTGAAGAG was identified by direct and reverse sequencing (Fig. 4A). Identical segment of III-2 has served as a wild type control, because no mutation was found there. (Fig. 4B).

By SSCP technique we have detected extra bands in

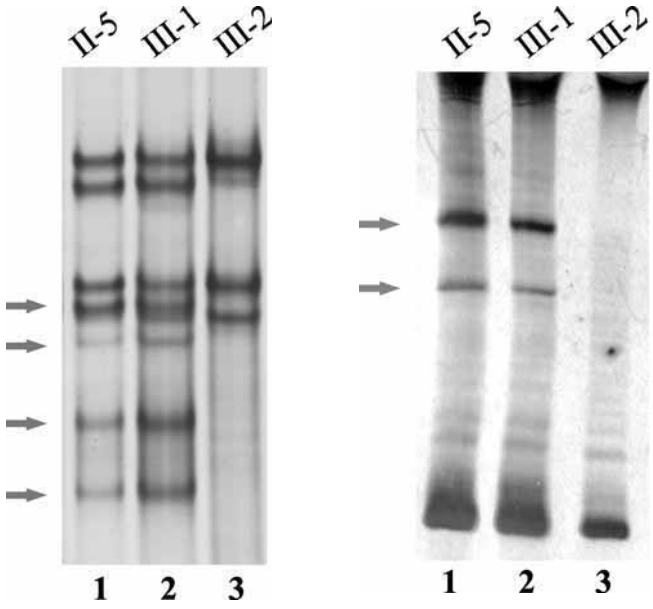

Figure 2. Members of analyzed family screened by SSCP technique for exon 11J of the BRCA1 gene. There are visable extra bands in lines 1 (proband, II-5) and 2 (proband's first daughter, III-1) that represent putative mutation in these samples (arrows). Bands' profile in line 3 (second daughter, III-2) doesn't show any abnormal configuratiion due to a mutation.

Figure 3. Heteroduplex analysis of genomic DNA samples of the proband (II-5) and her two daughters (III-1; 2). The formation of heteroduplexes (arrows) is seen in line 1 (proband, II-5) and 2 (proband's first daughter, III-1). Analogous heteroduplexes were not found in the sample of her second daughter III-2 (line 3).

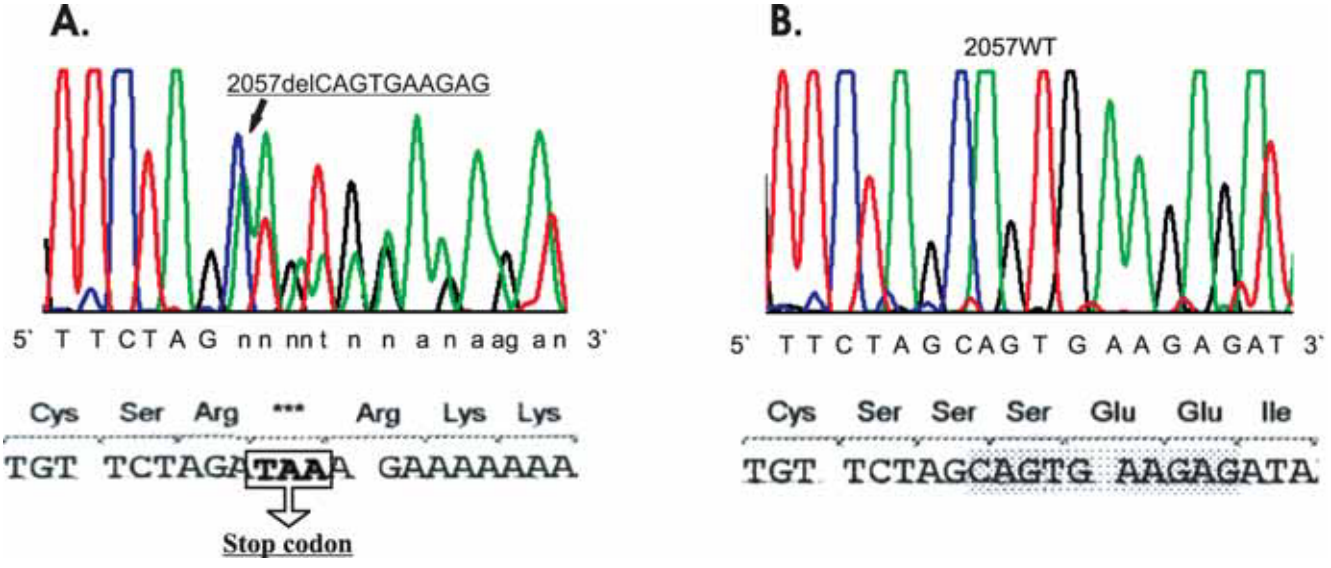

Figure 4. A. Confirmation of the novel BRCA1 mutation in proband's sample (II-5). Deletion of 10 base pairs (2057delCAGTGAAGAG) was identified by automatic sequencing (arrow). The novel stop codon TAA is generated by this deletion. B. Chromatogram of screened fragment without any mutation (III-2).

exons 2, 4, 11,12, 13 and in regions C, D, E, G, L, P of exon 15 of the APC gene in samples II-5 and III-1. Sequencing of these SSCP positive fragments has not revealed any additional mutation. Similarly, no mutations were detected in exons 5 and 7/8 of the TP53 gene and exons 1, 2 of K-ras oncogene (these data are not presented).

Discussion

Approximately 7% of breast cancer cases and 10% of ovarian cancer cases are thought to be associated with an autosomal dominant pattern of inheritance, induced by mutations in the BRCA1 and BRCA2 genes. The presence of a germline mutation in one of these genes increases the risk for breast and ovarian cancer. The majority of BRCA mutations are unique, that means each family with defined history of cancer tends to have its own mutation. On the other hand, a number of mutations common in defined populations such as Ashkenazi Jewish families have been identified [15].

Small number of cases of hereditary breast cancer may be attributed to rare genetic cancer predispositions including the Li-Fraumeni syndrome (TP53 gene), the Peutz-Jegher syndrome (STK11 gene) and the Cowden syndrome (PTEN gene) as well [2, 9, 5]. Causes of hereditary ovarian cancer include mutations in the DNA mismatch repair genes, primarily seen in association with hereditary non-polyposis colon cancer and possibly other genes, too.

Understanding the functions of the proteins encoded by the BRCA genes has provided insight how mutations in these genes increase the risk of cancer. Both BRCA1 and BRCA2 proteins prevent cells from becoming malignant by helping to repair mutations occured in other genes. This role is caused by the interactions of these proteins with proteins involved in DNA repair, particularly RAD51 [14, 16]. Moreover, both BRCA1 and BRCA2 proteins interact with transcriptional factors controlling the cell cycle [11, 13]. Mutational break in one of these genes may induce cancer by affecting their functions on different levels.

In this report we describe a new BRCA1 germline mutation in extremely seriously affected family. The SSCP technique was used for primal mutation screening. We revealed abnormal bands' profile that suggested the presence of a mutation (Fig. 2). The HDA confirmed results obtained by SSCP (Fig. 3). Sequencing of this suspected region of the BRCA1 gene (Fig. 4A) allowed identifying the novel germline mutation – deletion of 10 nucleotides (CAGTGAAGAG). This mutation has not been described so far. The new stop codon TAA is created directly at the place of deletion, which produces significantly truncated BRCA1 protein. Such truncated protein has lost most of its physiological functions, mentioned above.

We have been interested what role the mutations in other tumor-supressor genes or oncogenes play in promotion of different types of cancer in observed high risk family. For elucidation of strong pathogenic phenotype in this family we attempted to screen mutations in other genes which are very frequently impaired in cancer – TP53, K-ras and APC genes. SSCP technique showed extra bands in some exons or segments of exon 15 of the APC gene but it wasn't confirmed by sequencing of these SSCP positive fragments. Extra bands were detected as polymorphisms in some cases; having no pathogenic effect. We haven't detected any additional mutations in highly mutated exons of the TP53 gene and K-ras oncogene by both techniques as well. Severe pathologic disorders in this family seem to be caused by occurence of revealed novel germline mutation in the BRCA1 gene or by disruption of the function of other genes by somatic mutation. These alternatives will be studied whereas our screening of BRCA1 and also BRCA2 susceptibility genes is completely established for whole country.

However, identification of new mutation in mother and one of her daughter suggests that genetic testing plays an important role not only in elucidation of cancer process, but also in a prediction of disease progress and therapy improvement in mutation-carriers.

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