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ORIGINAL ARTICLE

Novel *BRCA1* splice-site mutation in ovarian cancer patients of Slavic origin

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Abstract Mutations in breast cancer susceptibility gene 1 (*BRCA1*) lead to defects in a number of cellular pathways including DNA damage repair and transcriptional regulation, resulting in the elevated genome instability and predisposing to breast and ovarian cancers. We report a novel mutation LRG_292t1:c.4356delA,p.(Ala1453Glnfs*3) in the 12th exon of *BRCA1*, in the splice site region near the donor site of intron 12. It is a frameshift mutation with the termination codon generated on the third amino acid position from the site of deletion. Human Splice Finder 3.0 and MutationTaster have assessed this variation as disease causing, based on the alteration of splicing, creation of premature stop codon and other potential alterations initiated by nucleotide deletion. Among the most important alterations are frameshift and splice site changes (score of the newly created donor splice site: 0.82). c.4356delA was associated with two ovarian cancer cases in two families of Slavic origin. It was detected by next generation sequencing, and confirmed with Sanger sequencing in both cases. Because of the fact that it changes the reading frame of the protein, novel mutation c.4356delA p.(Ala1453Glnfs*3) in *BRCA1* gene might be of clinical significance for hereditary ovarian cancer. Further functional as well as segregation analyses within the families are necessary for appropriate clinical classification of this variant. Since it has been detected in two ovarian cancer patients of Slavic origin, it is worth investigating founder effect of this mutation in Slavic populations.

Keywords *BRCA1* · Novel mutation · Ovarian cancer · Slavic populations

Introduction

More than one-fifth (about 23%) of ovarian carcinomas have been related to hereditary conditions, and in about 65–85% of those cases the genetic change is pathogenic germline mutation in *BRCA1/2* genes [1–5]. The life-time risk for ovarian cancer associated with *BRCA1* mutations is estimated to 43–76%, while the risk associated with *BRCA2* mutations is much lower—about 16.5% (7.5–34.0%) [6, 7]. On the other hand, ovarian cancer patients with *BRCA1/2* mutations have better prognosis than the patients without these mutations, they also have better response to platinum chemotherapy and can be eligible for therapy with PARP inhibitors [8–10]. The presence of *BRCA1/2* mutations might be considered as advantage for ovarian cancer patients.

About 50% of disease causing mutations are estimated to be *cis*-acting mutations that affect RNA splicing and lead to altered protein translation [11]. According to the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php), single nucleotide variants within the splice sites constitute about 10% of all mutations causing human inherited diseases. This number is probably even higher since

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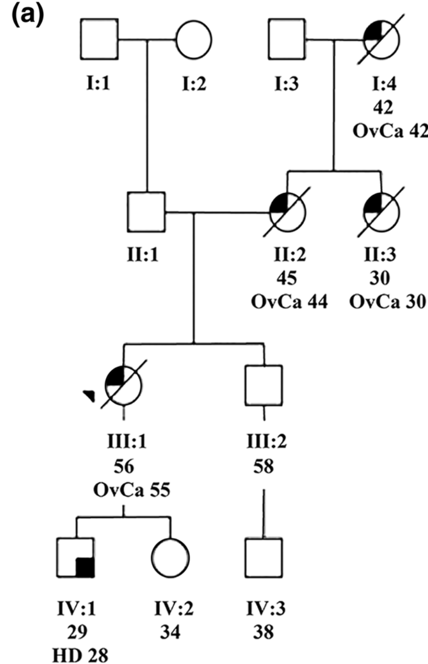
other types of mutations such as small insertions/deletions, nonsense mutations as well as synonymous mutations might affect the splicing efficiency [12]. When splice-site is abolished by mutation, the next available legitimate splice-site will be employed (exon skipping) or illegitimate splice-site in the vicinity will be used (cryptic splice-site utilization) [13]. Most of the splice site mutations that lead to human diseases involve the invariant GT and AG dinucleotides in the 5′ and 3′ splice sites [13].

This study presents a novel *BRCA1* splice-site pathogenic mutation found in two ovarian cancer patients in Serbia and Slovenia. This alteration is not described in the Breast Cancer Core Database (BIC), Human Gene Mutation Database (HGMD-Professional), Universal Mutation Database (UMD), Leiden Open Variation Database (LOVD) and NCBI ClinVar; nor has been documented in any published report to the best of our knowledge. Even though new technologies such as NGS have shifted the approach from targeted and recurrent gene mutation detection to whole gene or gene panel mutation detection, it is still of great importance to characterize mutations specific for particular populations. From the clinical point of view, recurrent mutations might contribute to targeted therapy development. Thanks to identification of recurrent/founder mutations it would be possible to develop specific diagnostics tests that will enable faster and cheaper mutation detection for the specific population. In the research area, identification of recurrent/founder mutations will help in estimation of genetic constitution, origin and relations among different populations.

Patients

In May, 2016, Serbian team has started to perform NGS for routine hereditary breast and ovarian cancer predisposition detection. Since then, we screened total of 146 subjects from Serbia for *BRCA1*/*2* mutations. Of those, 85 (58.2%) were ovarian cancer patients (selected and unselected for family history). Patients have been selected for testing according to national and international guidelines NCCN [14, 15]. Novel *BRCA1* splice-site pathogenic mutation was detected in only one ovarian cancer patient coming from family with the ovarian cancer burden (Family A) (Fig. 1a).

Family A (Serbia). Family history of the patient where mutation was detected (III:1) included three ovarian cancer cases. One of them was in first degree relative (II:2), and two in second degree relatives (II:3 and I:4) (Fig. 1a).



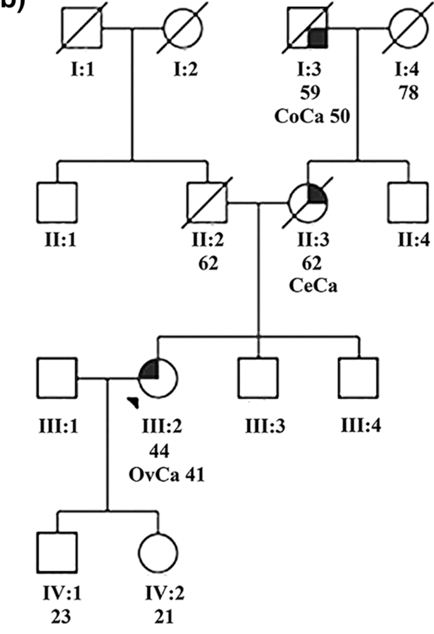


Fig. 1 Pedigrees of ovarian cancer families with novel *BRCA1* frameshift mutation. **a** Serbian family: *symbols with filled left upper quadrant* ovarian cancer; *symbols with filled right lower quadrant* Hodgkin disease. **b** Slovenian family: *symbols with filled right upper quadrant* cervix cancer; *symbols with filled left upper quadrant* ovarian cancer; *symbols with filled left lower quadrant* colorectal cancer. *Circles* are females, *squares* are males and diagonal *slash* indicates a deceased individual. ID numbers of individuals are below the *symbols*. The index subject is indicated by an arrow. *Ov Ca* ovarian cancer, *HD* Hodgkin disease, *Ce Ca* cervical cancer, *Co Ca* colorectal cancer. The numbers following these abbreviations indicate age at cancer diagnosis. The above written number is the current age, or the age of death

Family history also showed one case of Hodgkin disease in first degree relative (IV:1). The only additional blood sample available for targeted *BRCA1* mutation testing was from the daughter of the mutation carrier (IV:2). She was negative for novel c.4356delA *BRCA1* mutation. The blood samples, or the paraffin embedded tissue samples from the rest of the family members, were unavailable for further segregation analysis.

The Serbian patient was a 55-year-old post-menopausal woman diagnosed with ovarian cancer on both ovaries. At regular gynecological examination, abdominopelvic ultrasonography revealed solid masses on the ovaries, with no signs of fluid in the abdomen, and elevated serum CA-125 marker level (252 U/ml). A total abdominal hysterectomy with bilateral salpingo-oophorectomy and partial omentectomy was performed. The final diagnosis was stage IIIB serous adenocarcinoma. The patient received six cycles of paclitaxel plus carboplatin postoperative chemotherapy (conventional PC chemotherapy regimen). Follow-up abdominal and pelvic CT scans at the end of chemotherapy confirmed no evidence of disease. After 22 months, chest, abdominal and pelvic CT scans were notable for moderate-sized left pleural effusion, diffuse intraperitoneal metastatic deposits ("omental cake") and massive ascites. ECOG performance status was 2.

As the patient was disease-free for a period of 22 months (platinum-sensitive recurrent ovarian cancer), platinum-based chemotherapy was considered and carboplatin as a single agent was applied due to her poor general health condition. The patient had received three chemotherapy cycles. Despite applied chemotherapy and supportive therapy, the condition of the patient gradually deteriorated with an ECOG performance status 3–4. In this situation further chemotherapy was not considered, but only measures of palliative care. Secondary to relapsed ovarian cancer progression the patient died after 5 months.

In Slovenia, genetic counseling and testing of patients from families having a higher frequency of breast and/or ovarian cancer started in 1999. Since the end of 2014, NGS is in use for routine genetic screening. In the period from January 2015 until the end of December 2016, we have screened 247 ovarian cancer patients using NGS. Patients were selected for testing according to NCCN guidelines [14].

Family B (Slovenia). Mutation carrier (III:2) had developed ovarian cancer at 41 years of age (Fig. 1b). The mother of the carrier (II:3) was affected with cervical cancer. Carrier's third degree relative (I:3) had developed a colorectal cancer and was diagnosed at 50 years old. Daughter and son of the carrier were not tested for the presence of the novel c.4356delA *BRCA1* mutation nor other family members. The Slovenian patient was a 41-year old premenopausal woman who has previously been healthy. Her family history was unremarkable on her father's side. Her mother died of cervical carcinoma aged 62 and her maternal grandfather was diagnosed with colorectal carcinoma at the age of 50 and died aged 59.

The patient first presented with abdominal pain and weight loss. On initial evaluation an abdominal CT and ultrasound showed enlarged paraaortic lymph nodes with an increase in serum CA-125. A cervical biopsy performed due to vaginal bleeding showed what was considered to be a stage IV, poorly differentiated cervical carcinoma. Diffuse peritoneal carcinosis with numerous metastatic deposits was seen on diagnostic laparoscopy. Several samples were taken and showed the presence of a poorly differentiated serous carcinoma making the diagnosis of a high-grade serous ovarian adenocarcinoma stage IIIc most likely. As the disease was not resectable, patient underwent neoadjuvant chemotherapy and received four cycles of carboplatin in combination with paclitaxel. An excellent response to chemotherapy enabled her to undergo interval surgery. Hysterectomy and bilateral salpingo-oophorectomy in combination with an appendectomy and omentectomy was performed and optimal surgical cytoreduction was achieved. The patient received three applications of postoperative chemotherapy with additional applications of bevacizumab. On her last checkup, she showed no signs of relapse (disease free interval of 19 months). Due to her genetic testing results, she now has regular clinical breast exams and breast screening.

Methods

DNA was extracted from whole blood using InnuPREP Master Blood kit (Analytik Jena, Thuringia, D).

The coding sequence and exon/intron boundaries were enriched using Nextera DNA Library Preparation Kit in combination with TruSight® Cancer Panel (Illumina, San Diego, USA). Next generation sequencing was performed on Illumina MiSeq Sequencing System (Illumina) according to manufacturer's protocol. Secondary data analysis and base calling was performed by MiSeq Reporter Software 2.5.1, using DS Somatic as variant caller (Illumina). VCF v4.1 files generated during secondary analysis of sequencing data were imported into Illumina Variant Studio software for variant annotation and filtering.

Classification of detected variants was done through Illumina Variant Interpreter Software (Illumina).

Direct Sanger sequencing was performed to validate by NGS detected mutation in *BRCA1* gene in Serbian and Slovenian ovarian cancer patients [2, 16]. For direct DNA sequencing, the sample was bidirection-ally sequenced on an automated ABI PRISM 3130 and ABI 3500 genetic analyzers (Applied Biosystems, Foster City, CA).

In silico mutation prediction analysis was performed applying online bioinformatics tools MutationTaster2 and Human Splice Finder 3.0 [17, 18].

The total RNA from control sample negative for *BRCA1*:c.4356delA p.(Ala1453Glnfs*3) and mutated sample was extracted from whole blood using the miRNeasy Mini Kit (Qiagen, Hilden, Germany). The cDNA was synthetized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions and amplified using primer pairs flanking exons 10–14. The cDNA samples were sequenced by Sanger on ABI 3500 genetic analyzers (Applied Biosystems, Foster City, CA).

Results

Novel mutation was heterozygous deletion of a single adenine nucleotide in the 12th exon of *BRCA1* gene (LRG_292t1; chr17:41234422). The genetic alteration is located in the splice site region near the donor site of intron 12 in *BRCA1* (LRG_292t1: c.4356delA p.(Ala1453Glnfs*3); Fig. 2.) It is a frameshift mutation with the termination codon generated on the third amino acid position from the site of deletion (Fig. 3). Human Splice Finder 3.0 has predicted alteration of splicing, due to alteration of the wild type donor site, activation of an exonic cryptic donor site, creation of an exonic splicing silencer site and alteration of an exonic splicing enhancer site.

MutationTaster, has assessed the c.4356delA p.(Ala1453Glnfs*3) variation in *BRCA1* as disease causing, predominately based on creation of premature stop codon and different other potential alterations initiated by nucleotide deletion (Model: *complex_aae*, prob: 1). Among the most important alterations, are frameshift and splice site changes (score of the newly created donor splice site: 0.82).

Sequencing of mRNA revealed that mutation *BRCA1*:c.4356delA p.(Ala1453Glnfs*3) does not affect the donor site of intron 12. Except the deleted nucleotide at the position 4356, there was no additional difference between the control sample and mutated sample. The cDNA reversely transcribed from mRNA corresponded to reference sequence NM_007300.3 (Fig. 4).

Discussion

A number of risk factors have been associated with an ovarian cancer including early menarche, late menopause and low parity. On the contrary, each pregnancy results in about 13–19% reduction in risk [19]. Similarly, contraceptive pills have been demonstrated to have a protective

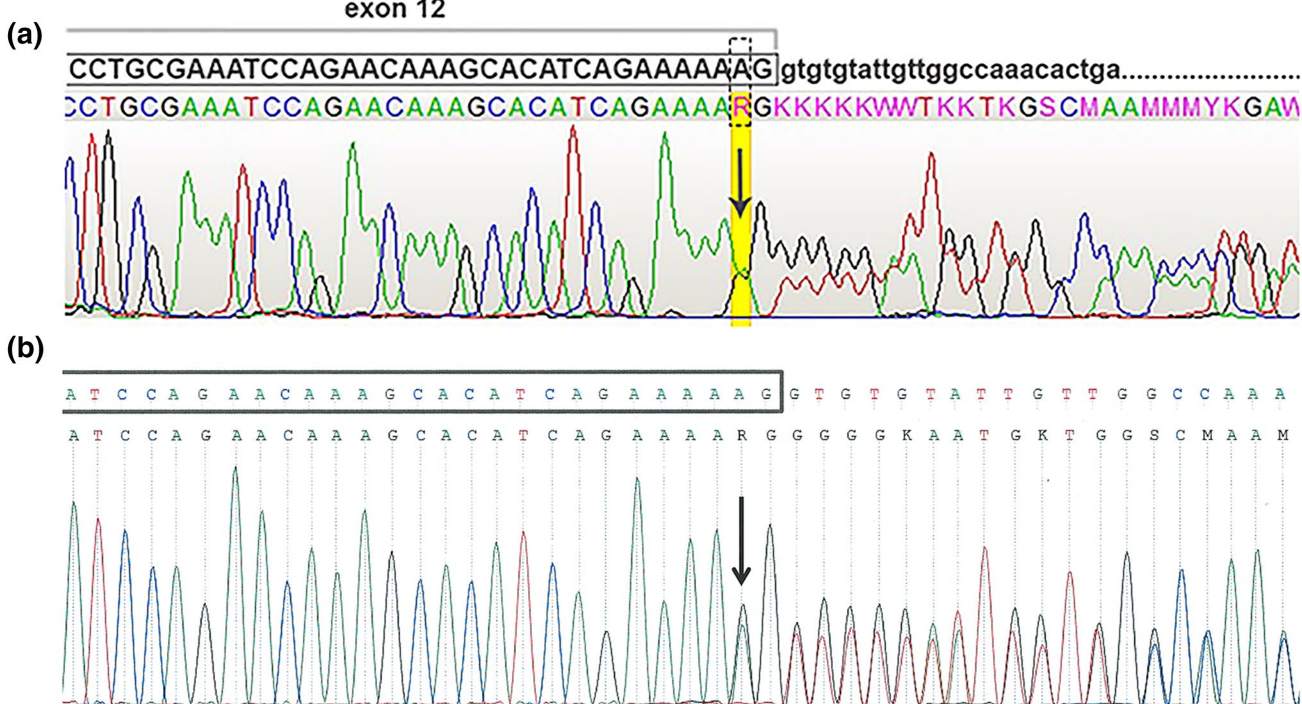
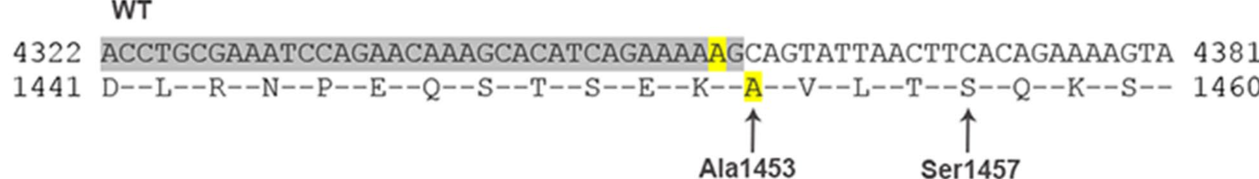


Fig. 2 Sequencing diagrams of a novel frameshift mutation c.4356delA p.(Ala1453Glnfs*3) detected in the *BRCA1* gene in patient with ovarian carcinoma from **a** Serbia and **b** Slovenia. The deletion site is indicated by the *arrow*



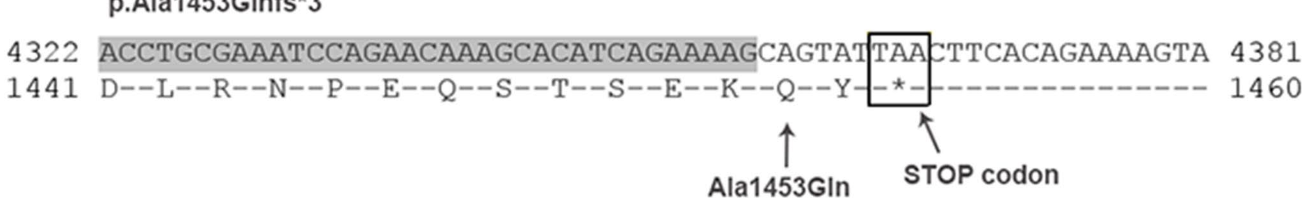


Fig. 3 Deletion of a single nucleotide causes the shift in the reading frame of the BRCA1 protein and occurrence of STOP codon on the third position from the deletion site

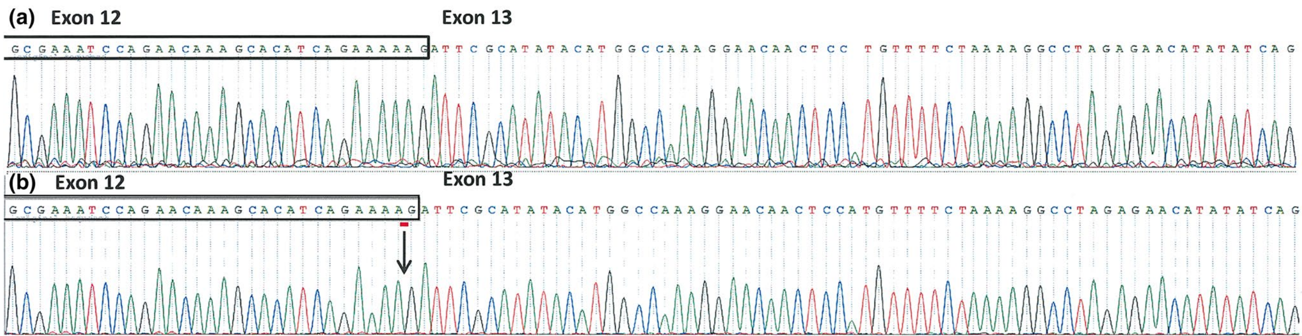


Fig. 4 Sanger sequencing of a control and mutated cDNA; **a** control cDNA (wild type), displaying the canonic junction between exons 12 and 13 of the *BRCA1* gene. **b** Mutated patient's cDNA, bearing the *BRCA1*:c.4356delA splice variant (*black arrow*) revealing the unchanged canonic splice site and junction between exons 12 and 13 of the *BRCA1* gene

effect which even persists for many years after the pill has been stopped [20]. Besides mentioned risks factors, family history of ovarian cancer is the strongest risk factor for this disease [21]. Life-time risk for developing ovarian cancer in the general population is 1.6%, but the risk is higher for women with family history of disease. Women with one first-degree relative with ovarian cancer have an approximately 5% risk while those with two first-degree relatives with ovarian cancer have a 7% risk [22].

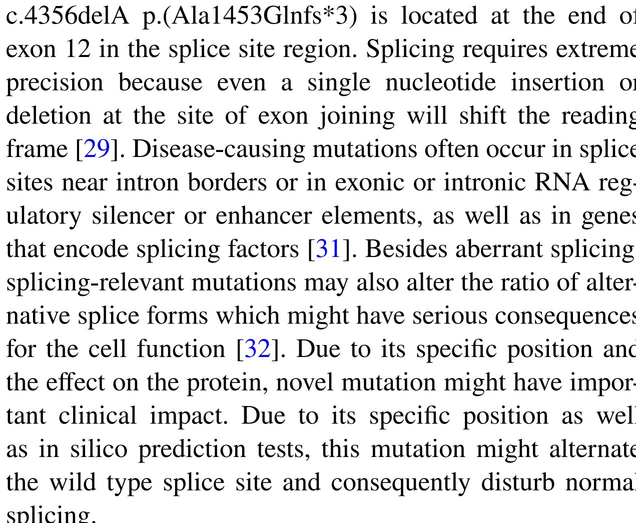
The majority of familial ovarian cancers are related to hereditary breast and/or ovarian cancer syndrome (HBOC), yet the lesser proportion is related to other hereditary syndromes, such as Lynch and Peutz-Jeghers syndromes [23, 24]. Accordingly, the majority of women with inherited ovarian cancers carry a deleterious mutation in one of two susceptibility genes, *BRCA1* or *BRCA2* [25] or less frequently, other susceptibility genes such as *MSH2, MLH1, MSH6, BRIP1, RAD51C, RAD51D* and *STK11* [14].

According to the Breast Cancer Information Core database, more than 1500 mutations have been detected in *BRCA1* gene. Of these, around 800 have been classified as pathogenic or clinically significant, which means that their effects on the protein function and the cancer risk have been proven. According to the same database, mutations associated with cancer cluster frequently in three domains of *BRCA1* gene. These domains are: RING domain (exons 2–7), a region coded by the exons 11–13 and the BRCT domain (exons 16–24). Exons 11–13 together cover over 65% of the *BRCA1* sequence. Exon 11 encodes two nuclear localization sequences (NLS) and binding sites for several proteins including retinoblastoma protein (Rb), cMyc, Rad50 and Rad51. Amino acids encoded by the exons 12 and 13 contain domain which mediates interaction with

PALB2 protein as well as part of serine containing domain which is phosphorylated by the ATM [26]. Proteins that binds to the exon 11–13 encoded peptides are involved in a wide range of cellular pathways such as suppression of cell cycle (Rb), NHEJ (Rad50, Rad51), transcription regulation (Myc), HR (PALB2), nuclear localization and phosphorylation (by ATM, ATR, Chek2). Mutations that occur in this part of the gene might affect tumor suppressor function of BRCA1 leading to increased risk for cancer development.

Novel mutation detected in two ovarian cancer cases from Serbia and Slovenia is located at the end of exon 12, in the part of the *BRCA1* gene that encodes Serine Cluster Domain (SCD) of the protein. This domain spans amino acids from 1280 to 1524 and it is phosphorylated by ATM and ATR kinases which are activated by the DNA damage [26]. Phosphorylation of BRCA1 triggers recruitment of BRCA1 to sites of double strand breaks and repair initiation. Serine residues 1423 and 1524 are redundantly phosphorylated by ATM and ATR, whereas S1387 is a specific target for ATM and S1457 for ATR [27]. Mutations of serine residues may affect localization of BRCA1 to sites of DNA damage and DNA damage response function [28]. Mutation detected in this study, c.4356delA p.(Ala1453Glnfs*3), does not affect serine residues directly. By the scenario that primary splice site remains unaffected, the frameshift would create amino acid change on the 1453 position and termination codon on the 1455 amino-acid position. The mRNA carrying the premature stop codon might go through a nonsense-mediated decay (NMD) pathway, leading to the elimination of aberrant mRNA, or amino acid sequence change resulting in affected protein features. However, this mutation leads to premature ending in protein synthesis and to the production of truncating protein at this position. The lack of important serine residues in the truncated protein might lead to inadequate BRCA1 phosphorylation and its inability to interact with partner proteins in the promotion of DNA repair pathways.

With the greater understanding of gene expression regulation, it has become clear that RNA is much more than a passive intermediate between the DNA and the proteins. The control of RNA processing is now recognized as a crucial component of gene expression regulation [29]. The process of splicing entails the recognition of various motifs including consensus sequences flanking the GT and AG dinucleotides at the 5′ (donor) and 3′ (acceptor) splice-sites, as well as the motif located 15–35 base pairs upstream of the 3′ splice-site [30]. By the second scenario, besides the effects that novel mutation c.4356delA p.(Ala1453Glnfs*3) in *BRCA1* might have on the phosphorylation and adequate translocation of BRCA1 protein to the DNA damage site, the deletion of a single nucleotide in this position might also affect BRCA1 mRNA splicing. Novel mutation



c.4356delA p.(Ala1453Glnfs*3) is located at the end of exon 12 in the splice site region. Splicing requires extreme precision because even a single nucleotide insertion or deletion at the site of exon joining will shift the reading frame [29]. Disease-causing mutations often occur in splice sites near intron borders or in exonic or intronic RNA regulatory silencer or enhancer elements, as well as in genes that encode splicing factors [31]. Besides aberrant splicing, splicing-relevant mutations may also alter the ratio of alternative splice forms which might have serious consequences for the cell function [32]. Due to its specific position and the effect on the protein, novel mutation might have important clinical impact. Due to its specific position as well as in silico prediction tests, this mutation might alternate the wild type splice site and consequently disturb normal splicing.

Intending to clarify what is the true implication of the detected alteration *BRCA1*:c.4356delA p.(Ala1453Glnfs*3), the RNA was isolated from Slovene patient and from control sample negative for this specific mutation and subjected to direct sequencing by Sanger. The sequencing results have clearly shown that the detected alteration did not affect the primary splice site. Actually, the *frameshift* resulted in amino acid change on the 1453 position and creation of the termination codon on the 1455 amino-acid position, which leads to premature termination in protein synthesis and to production of truncated protein.

Because of the fact that it changes the reading frame of the protein, novel mutation c.4356delA p.(Ala1453Glnfs*3) in *BRCA1* gene might be of clinical significance for hereditary ovarian cancer. Further functional as well as segregation analyses within the families are necessary for appropriate clinical classification of this variant. Since it has been detected in two ovarian cancer patients of Slavic origin, it would be important to expand the size of the investigated group of patients in order to determine the possible founder effect of this mutation in Slavic populations.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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