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Identification of a novel mutations *BRCA1**c.80 + 3del4 and *BRCA2**c.6589delA in Slovak HBOC families

Michal Konecny · Miriam Vizvaryova · Katarina Zavodna · Regina Behulova · Maria Gerykova Bujalkova · Tomas Krivulcik · Frantisek Cisarik · Juraj Kausitz · Eva Weismanova

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Abstract Mutations in the *BRCA1* and *BRCA2* genes account for the majority of hereditary breast ovarian cancer (HBOC) cases. However, after *BRCA1* and *BRCA2* screening still the most HBOC cases remain negative for any mutational event. Accordingly, in these cases raises the relevance to analyze the unusual *BRCA1/2* variants of uncertain clinical significance. Complex RNA/cDNA analysis may constitute the solution and help to interpret the HBOC syndrome in the family. In our study we analyzed the novel, to our knowledge, not yet published mutations identified in Slovak HBOC families, c.80 + 3del4 (IVS2 + 3delAGTC) in *BRCA1* gene and mutation c.6589delA (6817delA) in *BRCA2* gene. To determine the effect of the *BRCA1* mutation, we applied different approaches: segregation analysis of mutation with disease, presence in the set of unaffected controls and finally RNA/cDNA *BRCA1* analysis. Novel *BRCA2* mutation was determined performing direct sequencing analysis. In conclusion, considering the results from all used techniques we approved the

mentioned mutations as seriously pathogenic and disease causing with clear effect on the onset of HBOC syndrome.

Keywords *BRCA1* · *BRCA2* · Novel mutation · Splicing mutation · Hereditary breast ovarian cancer (HBOC) · Slovak population

Introduction

Germ-line mutations in *BRCA1* and *BRCA2* are responsible for the most of the hereditary breast and ovarian cancer (HBOC) cases, thus account for approximately 15–20% of breast and 60–80% of breast and ovarian cancer cases [1]. However, in all tested populations, the frequency of identified *BRCA1* and *BRCA2* mutations in high-risk HBOC families was consistently lower than predicted by linkage analysis [2].

Although, the part of the *BRCA1/BRCA2* negative cases (5–10%) may be assigned to the presence of PCR undetectable large genomic rearrangements (LGRs) [3], still the most HBOC cases remain negative for any mutational event.

Generally, germ-line mutations identified in *BRCA* genes may be divided according to their clinical significance into three groups: (1) disease-causing, pathogenic mutations (clear clinical consequence); (2) single nucleotide polymorphisms (SNPs), mostly missense or synonymous mutations (no clinical consequence) and (3) variants of uncertain significance (unknown clinical consequence) [4].

Intronic uncertain variants may lead to the aberrant splicing through affecting the intron consensus sequences at the splice sites, or through creating ectopic splice sites. Missense exonic uncertain variants may intervene the exonic splice enhancer (ESE) sites, sequences localized

M. Konecny (✉) · M. Vizvaryova · K. Zavodna · J. Kausitz · E. Weismanova
Department of Clinical Genetics, St. Elizabeth Cancer Institute, Heydukova 10, 812 50 Bratislava, Slovakia
e-mail: mkonecny@ousa.sk

R. Behulova
Institute of Medical Biology and Genetics, Faculty of Medicine, Comenius University, Bratislava, Slovakia

K. Zavodna · M. Gerykova Bujalkova · T. Krivulcik
Laboratory of Cancer Genetics, Cancer Research Institute of Slovak Academy of Sciences, Bratislava, Slovakia

F. Cisarik
Department of Clinical Genetics, Hospital and Clinic, Zilina, Slovakia

within exons that promote and regulate the splicing of the appropriate transcript [5] or influence the functions of protein domains.

Molecular evaluation of variants of uncertain significance is very demanding and a variety of approaches for their classification have been reported. These include RNA/cDNA analysis, co-segregation of the variant with disease in the family history, LOH in the tumors, the occurrence in the set of unaffected controls, co-occurrence of the variant with pathogenic mutation in the same gene, prediction based on the amino acid conservation and position in the protein, biochemical and structural assays and mouse models [6].

Recently, the splicing studies indicate that up to 70% of human genes may have alternatively spliced mRNA isoforms [7]. Thus far, three protein products of wild type *BRCA1* alternative splice variants have been identified (*BRCA1*-wt, *BRCA1*-del11B, *BRCA1*-IRIS) at different levels [8]. However recently Lixia et al. [9] reported nine *BRCA1* alternative variants in breast cancer cell line, indicating that *BRCA1* may have unexpectedly other splice variants.

Herein, we report two novel, to our knowledge, not yet published mutations detected in *BRCA1* and *BRCA2* genes. We demonstrate the disease causing effect of novel *BRCA1* mutation c.80 + 3del4 using the combination of segregation analysis and RNA/cDNA analysis. Concurrently, we show clear pathogenic effect of novel *BRCA2* frame-shift mutation c.6589delA creating stop codone leading to truncated protein.

Patients and methods

BRCA1 HBOC family

Healthy proband (III:2) from HBOC family was analyzed for presence of mutations in *BRCA1* gene. DNA was extracted from whole blood using QIAamp DNA Mini Kit (Qiagen). PCR reaction was performed using primers specific for the coding sequence and exon–intron boundaries of all *BRCA1* exons, which were analyzed using sequencing analysis with BigDye terminator v1.1 cycle sequencing kit on ABI 3130 genetic analyzer (applied biosystems). This analysis revealed the novel mutation c.80 + 3del4, which constitute the deletion of AGTC nucleotides three bases after exon 2, after the GT splice signal.

Patient II:2 (Fig. 1a), mother of the healthy proband (III:2) died from ovarian cancer diagnosed at the age of 66 years. Patient II:3 died from ovarian cancer diagnosed at the age of 52 years. Patient I:2 died from breast cancer diagnosed at the age of 59 years and her two sisters (I:3, I:4) died from breast, respectively ovarian cancer

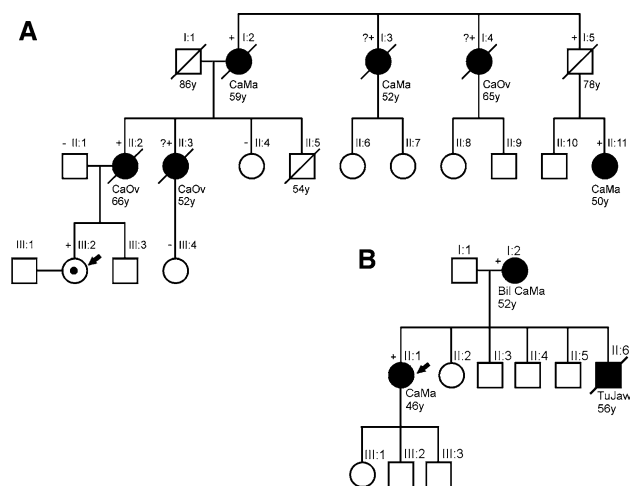


Fig. 1 a *BRCA1* family history; b *BRCA2* family history. The abbreviations: CaOv, ovarian cancer; CaMa, breast (mammary) cancer; y, age at diagnosis; +, presence of mutation; ?+, suspected presence of mutation; -, mutation not detected; arrow, primarily analyzed proband

diagnosed at the age of 52 and 65 years. Patient II:11 had diagnosed breast cancer at the age of 50 years.

In the next step, to exclude the possible neutral, or uncertain significance of the variant c.80 + 3del4, we analyzed the set of 200 unaffected controls using sequencing analysis of exon 2 of *BRCA1* gene.

Peripheral blood samples with EDTA from mutation carriers III:2 and II:11 and non-carriers II:1, II:4 and III:4 were collected in two steps. In the first step, blood from III:2, II:1 and II:4 was collected, leukocytes separated and stored at -20°C . Alternatively, in the second step, all blood samples were collected (II:1, II:4, II:11, III:2, III:4) and before RNA isolation treated with RNAlater solution (Ambion) for longer storage at -20°C .

Later, total RNA was isolated using RiboPure Blood kit (Ambion) and at the end of the isolation treated to avoid DNA contamination using DNase I (Ambion) according to manufacturer's recommendations. Isolated RNA was quantified on the Nanodrop N-1000 (Thermo Scientific) and qualitatively assessed in the RT-PCR reaction of the housekeeper gene (GAPDH) using primers GAPDH-S and GAPDH-A. The concentrations of RNA samples varied from 15 to 60 ng/ μl , with purity (OD 260/280) from 1,7 to 2,0 and no RNA sample was degraded. Wild type control sample was employed with each RNA isolation.

RT-PCR and PCR reactions were performed in single tube by One-Step RT-PCR Kit (Qiagen), according to manufacturer's protocol, using cDNA *BRCA1* primers C1AF: 5'-GCTGTGGGGTTTCTCAGATA-3', and C2AR: 5'-AAGTCTTTTGGCACGGTTTC-3', amplifying the region from exon 1A through exon 7, resulting in expected fragment of length 497 bp. After cDNA amplification, the

fragments were visualized by the agarose gel (2%) electrophoresis. The sequence of amplified PCR fragment was identified using direct sequencing analysis in both forward and reverse directions.

BRCA2 HBOC family

Patient with breast cancer diagnosed at the age of 46 years passed the analysis of all exons of *BRCA1* gene, which was based on SSCP analysis and sequencing of atypical PCR fragments. *BRCA1* analysis was finished as negative for presence of pathogenic mutation. Further the sample was analyzed for presence of mutations in *BRCA2* gene, especially exons 5, 6, 10, 11, 17, 20, 23, 25 were diagnosed. Theoretically, pathogenic mutations in these *BRCA2* exons are responsible for 89% of all *BRCA2* positive families in Central and Eastern Europe.

Analyzed patient had diagnosed breast cancer at the age of 46 years (II:1) (Fig. 1b). Mother (I:2) of the analyzed patient suffered bilateral breast cancer diagnosed at the age of 52 years. Brother (II:6) of the patient died from malign tumor of the face jaw at the age of 56 years. Patient has even three other healthy brothers and one healthy sister, also has one daughter and two sons.

Primarily, peripheral blood samples with EDTA from sample II:1 was collected and DNA was isolated using QIAamp DNA mini kit (Qiagen). PCR reaction was performed using primers specific for the coding sequence and exon–intron boundaries of mentioned *BRCA2* exons [10], and further analyzed using sequencing analysis with Big-Dye terminator v1.1 cycle sequencing kit on ABI 3130 genetic analyser (applied biosystems). The presented mutation in exon 11 PCR fragment was identified reading sequencing results in both forward and reverse directions and confirmed by repeated analysis from independent sample offtake. After mutation detection, peripheral blood from sample I:2 was collected, DNA extracted and PCR of exon 11 fragment, carrying the mutation was performed.

Results

The novel *BRCA1* variant c.80 + 3del4 (IVS2 + 3delA-GTC) was detected in healthy proband III:2 and in living breast/ovarian cancer patient II:11 and was not found in proband II:1, nor in proband II:4 (Fig. 1a). These results directly demonstrated that the variant is inherited from the patient II:2 and very presumably segregates with HBOC disease. Moreover, the analysis of the presence of variant c.80 + 3del4 in the set of 200 unaffected controls revealed no detection.

Primarily, RNA was isolated from samples III:2, II:1 and II:4 stored at -20°C and not treated with *RNAlater*

solution. The quantification of RNA showed concentrations from 15 to 32 ng/ μl . RT-PCR and PCR reaction successfully amplified 393 bp cDNA fragment of exon 1A-7 only in sample III:2, the carrier of the c.80 + 3del4 variant. The direct sequencing analysis and analysis using BLAST search [11] revealed the presence of fragment, which concerned exon 1A, 3, 5, 6, 7 however lacked exon 2 and also 6 bases (GTAAAG) from the end of exon 1A (Fig. 2c).

Secondary, the RNA analysis continued with samples II:1, II:4, II:11, III:2, III:4 treated with *RNAlater* solution and stored at -20°C . Isolated RNA was quantified (concentrations from 17 to 60 ng/ μl) and RT-PCR reaction was performed. In non-carriers of the c.80 + 3del4 variant (II:1, II:4, III:4) and wild-type control the presence of 498 bp fragment was detected on the agarose gel. However, gel electrophoresis in the c.80 + 3del4 variant carrier samples (III:2, II:11) showed presence of minimally two fragments, shorter fragment (mutant) with stronger signal and longer one with lower signal (wild type) (data not shown).

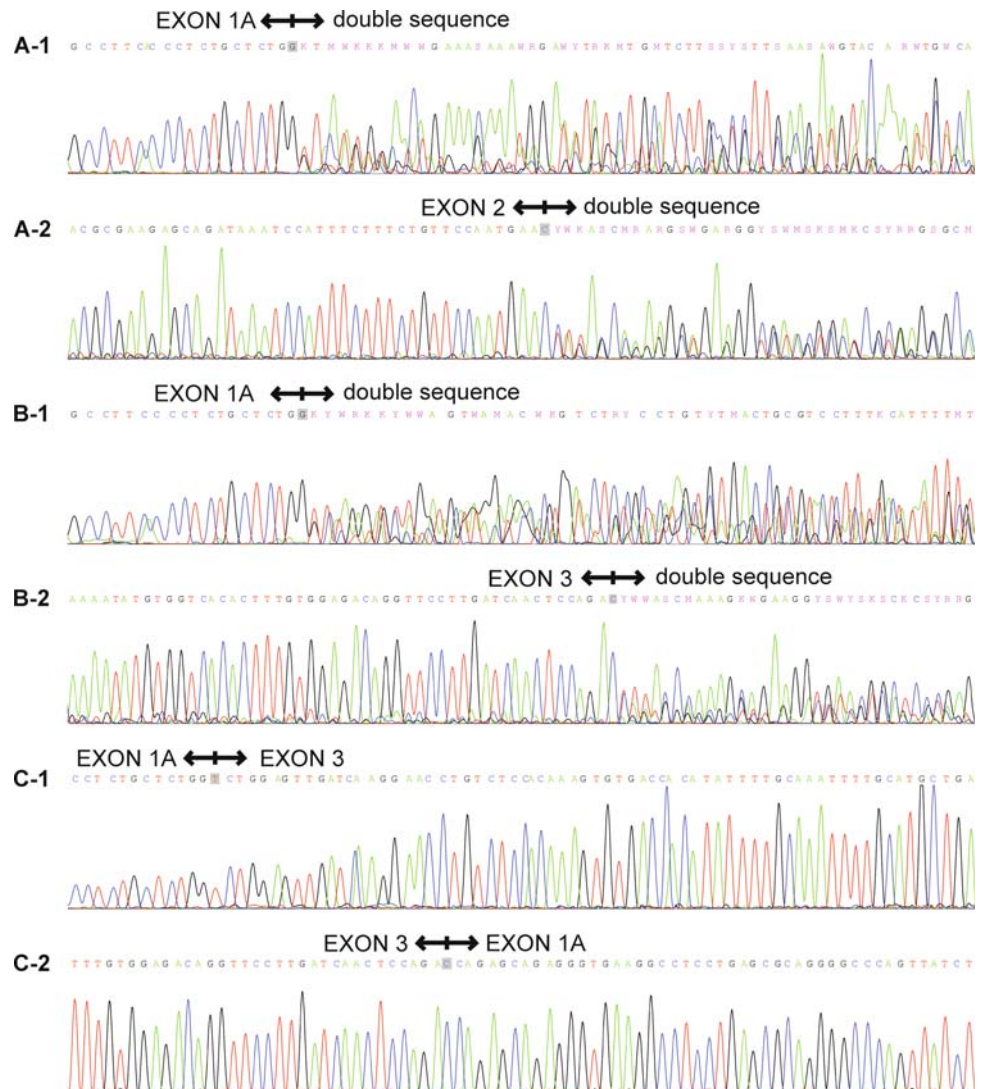
In non-carriers and wild-type samples sequencing analysis together with BLAST search [11] showed presence of two fragments with difference of 6 bp. The first fragment consisted of exons 1A, 2, 3, 5, 6, 7; the second fragment comprised same exons but lacked 6 bases (GTAAAG) from the end of exon 1A (Fig. 2a). Sequencing analysis of cDNA from both directions (forward, reverse) in variant carriers revealed the presence of cDNA fragment comprised all exons 1A, 2, 3, 5, 6, 7 but also fragment consisted of exons 1A, 3, 5, 6, 7, with deleted exon 2 (Fig. 2b). The sequence analysis of cDNA fragment (exons 1A-7) from both directions confirmed that the novel *BRCA1* mutation c.80 + 3del4 results in the complete skipping of exon 2.

The novel *BRCA2* mutation c.6589delA was primarily detected in patient II:1 (Fig. 1b) performing direct sequencing analysis. Sequencing results revealed presence of double overlapping sequence from position 6817 (Genebank U43746.1) from forward direction. From reverse direction double sequence caused by deletion of T was localized to the same position 6817. Later the presence of deletion was also confirmed by targeted sequencing analysis of exon 11 of *BRCA2* gene in patient's mother (I:2). Mutation c.6589delA was clearly localized to position 6,817, affecting codone for Thr at position 2197 in exon 11 of *BRCA2* protein.

Discussion

Variants of unclassified significance (intronic or missense exonic variants) are prevalently studied only at genomic DNA level, thus their potential disease-associated effect cannot be discovered. We demonstrated, by performing the

Fig. 2 Sequencing analysis electropherograms of the cDNA samples, using PCR primers [C1AF; C2AR] localized at the beginning of exon 1A and at the end of exon 7. **a-1** Sequencing analysis of the mutation c.80 + 3del4 non-carrier sample, forward direction. **a-2** Sequencing analysis of the mutation c.80 + 3del4 non-carrier sample, reverse direction. **b-1** Sequencing analysis of the mutation c.80 + 3del4 carrier sample, forward direction. **b-2** Sequencing analysis of the mutation c.80 + 3del4 carrier sample, reverse direction. **c-1** Sequencing analysis of the mutation c.80 + 3del4 carrier sample, forward direction. RNA/cDNA from sample from white-cells fraction not treated with *RNAlater* solution and stored at -20°C . **c-2** Sequencing analysis of the mutation c.80 + 3del4 carrier sample, reverse direction. RNA/cDNA from sample from white-cells fraction not treated with *RNAlater* solution and stored at -20°C



cDNA sequencing analysis, that novel intronic variant c.80 + 3del4 (IVS2 + 3delAGTC) affects the *BRCA1* splicing.

The sequencing analysis of cDNA fragments was embarrassed by presence of more than one fragment (exons 1A-7 and exons 1A-7 with deletion of GTAAAG) because of alternative *BRCA1* splicing. This alteration in splicing was reported by Lixia et al. [9] in breast cancer cell lines, however we detected the alterations in non-carrier and wild-type samples. We consider, that this alternative variant may represent a wild-type *BRCA1* isoform and may be tissue specific. The problem of *BRCA1* alternative splicing at the beginning of cDNA (exon 1A and 2) should be a subject of further analysis.

In c.80 + 3del4 mutation carrier samples treated with *RNAlater* solution, the cDNA sequencing analysis also revealed more fragments, however reading the sequences from both directions (forward, reverse) identified the complete deletion of exon 2. Surprisingly, in the sample of

mutation carrier (white-cells fraction) stored at -20°C without *RNAlater* solution we were able to detect single fragment consisted of exons 1A-7 with deleted exon 2 and either GTAAAG from the 3' end of 1A. These findings led us to the speculation that the fragment with deleted exon 2 might be in quantitative abundance in analyzed tissue and thus was not degraded during the storage period, while the second fragment did. Nevertheless the problems with presence of more PCR fragments, sequence analysis based on the both direction reading was able to identify the sequence of our interest, respectively the positions of deletion's start. Generally, according to the mentioned cDNA sequencing results we may summarize, that in wild type and non-carrier samples we have detected normal expected cDNA fragment comprising exons 1A-7. On the contrary, in c.80 + 3del4 mutation carrier samples we have identified cDNA fragment including exons 1A and exon 3 to exon 7, with deletion of exon 2. We assume that in this case there might be also other fragments with or

without GTAAAG at the end of exon 1A, though cannot be distinguished by used method and might be in a very low concentration.

Theoretically, the complete deletion of exon 2 excludes 47 amino acid residues, even the initial Met residue at the position 120 (according to U14680). Therefore, the translation of the aberrant protein would start at the next Met residue (ATG), which is in the position 261 in exon 5 and leads to the synthesis of C-terminally truncated BRCA1 protein. Such amino acid deletion would detached first 47 amino acid residues, together with 5 Cys residues (at amino acid positions 24, 27, 39, 44 and 47) from RING finger domain and thus would influence and presumably seriously interrupt the protein function. The RING finger domain is defined by a conserved pattern of Cys residues that is found in a wide variety of different proteins. The importance of the RING finger domain (aa residues 24–64) is supported by the observations, that the first 100 residues is the most highly conserved region in *BRCA1* gene [12]. The RING domains mediate interaction between BRCA1 and BARD1 proteins [13], and form heterodimer functioning as a potent E3 ubiquitin ligase [14]. In the monoubiquitination of histones appears to affect transcription and chromatin remodeling [15].

Novel frame-shift mutation c.6589delA in exon 11 of *BRCA2* gene in analyzed family clearly segregates with disease. Mutation renders codone for Thr at position 2197 and causes frame shift and generation of aberrant stop codone at position 2204 that leads to creation of the truncated protein. The aberrant stop codone interfere the BRC repeat region in exon 11, which is essential for binding with RAD51 protein and thus for DNA repair. The *BRCA2* protein truncation also causes absence of C-terminal conserved region basic for bound with DSS1 and DNA binding process [16].

In summary, the combination of different approaches: segregation analysis of mutation c.80 + 3del4 with disease within the described family, absence of the variant in the set of unaffected controls and RNA/cDNA analysis led us to the conclusion, that novel variant c.80 + 3del4 seriously affects *BRCA1* splicing and thus should be considered as disease-causing. DNA analysis based on the direct sequencing analysis of *BRCA2* gene revealed novel frame-shift mutation c.6589delA in exon 11 possible specific for Slovak HBOC population.

References

- Gayther SA, de Foy KA, Harrington P, Pharoah P, Dunsmuir WD, Edwards SM, Gillett C, Ardern-Jones A, Dearnaley DP, Easton DF, Ford D, Shearer RJ, Kirby RS, Dowe AL, Kelly J, Stratton MR, Ponder BA, Barnes D, Eeles RA (2000) The frequency of germ-line mutations in the breast cancer predisposition genes *BRCA1* and *BRCA2* in familial prostate cancer. The Cancer Research Campaign/British Prostate Group United Kingdom Familial Prostate Cancer Study Collaborators. *Cancer Res* 60:4513–4518
- Ford D, Easton DF, Stratton M, Narod S, Goldgar D, Devilee P et al (1998) Genetic heterogeneity and penetrance analysis of the *BRCA1* and *BRCA2* genes in breast cancer families. *Am J Hum Genet* 62:676–893. doi:10.1086/301749
- De la Hoya M, Gutiérrez-Enríquez S, Velasco E, Osorio A, Sánchez de Abajo A, Vega A, Salazar R, Esteban E, Llort G, Gonzalez-Sarmiento R et al (2006) Genomic rearrangements at the *BRCA1* locus in Spanish families with breast/ovarian cancer. *Clin Chem* 52:1480–1485. doi:10.1373/clinchem.2006.070110
- Breast Cancer Information Core (BIC) <http://research.nhgri.nih.gov/bic/>. Accessed 29 Oct 2008
- Chenevix-Trench G, Healey S, Lakhani S, Waring P, Cummings M, Brinkworth R, Deffenbaugh AM, Burbidge LA, Pruss D, Judkins T, Scholl T, Bekessy A, Marsh A, Lovelock P, Wong M, Tesoriero A, Renard H, Southey M, Hopper JL, Yannoukakos K, Brown M, Easton D, Tavtigian SV, Goldgar D, Spurdle AB, kConFab Investigators (2006) Genetic and histopathologic evaluation of *BRCA1* and *BRCA2* DNA sequence variants of unknown clinical significance. *Cancer Res* 66(4):2019–2027. doi:10.1158/0008-5472.CAN-05-3546
- Billack B, Monteiro AN (2004) Methods to classify *BRCA1* variants of uncertain clinical significance: the more the merrier. *Cancer Biol Ther* 3(5):458–459
- Kalnina Z, Zayakin P, Silina K, Linē A (2005) Alterations of pre-mRNA splicing in cancer. *Genes Chromosomes Cancer* 42(4):342–357. doi:10.1002/gcc.20156
- Fortin J, Moisan AM, Dumont M, Leblanc G, Labrie Y, Durocher F, Bessette P, Bridge P, Chiquette J, Laframboise R, Lépine J, Lespérance B, Pichette R, Plante M, Provencher L, Voyer P, Simard J (2005) A new alternative splice variant of *BRCA1* containing an additional in-frame exon. *Biochim Biophys Acta* 1731(1):57–65
- Lixia M, Zhijian C, Chao S, Chaojiang G, Congyi Z (2007) Alternative splicing of breast cancer associated gene *BRCA1* from breast cancer cell line. *J Biochem Mol Biol* 40(1):15–21
- Wagner TM, Hirtenlehner K, Shen P, Moeslinger R, Muhr D, Fleischmann E, Concin H, Doeller W, Haid A, Lang AH, Mayer P, Petru E, Ropp E, Langbauer G, Kubista E, Scheiner O, Underhill P, Mountain J, Stierer M, Zielinski C, Oefner P (1999) Global sequence diversity of *BRCA2*: analysis of 71 breast cancer families and 95 control individuals of worldwide populations. *Hum Mol Genet* 8(3):413–423. doi:10.1093/hmg/8.3.413
- BLAST <http://blast.ncbi.nlm.nih.gov/Blast.cgi>
- Meza JE, Brzovic PS, King MC, Klevit RE (1999) Mapping the functional domains of *BRCA1*. Interaction of the ring finger domains of *BRCA1* and *BARD1*. *J Biol Chem* 274(9):5659–5665. doi:10.1074/jbc.274.9.5659
- Brzovic PS, Rajagopal P, Hoyt DW, King MC, Klevit RE (2001) Structure of a *BRCA1-BARD1* heterodimeric RING-RING complex. *Nat Struct Biol* 8(10):833–837. doi:10.1038/nsb1001-833
- Hashizume R, Fukuda M, Maeda I, Nishikawa H, Oyake D, Yabuki Y, Ogata H, Ohta T (2001) The RING heterodimer *BRCA1-BARD1* is a ubiquitin ligase inactivated by a breast cancer-derived mutation. *J Biol Chem* 276(18):14537–14540. doi:10.1074/jbc.C000881200
- Bach I, Ostendorff HP (2003) Orchestrating nuclear functions: ubiquitin sets the rhythm. *Trends Biochem Sci* 28(4):189–195. doi:10.1016/S0968-0004(03)00055-0
- Jasin M (2002) Homologous repair of DNA damage and tumorigenesis: the BRCA connection. *Oncogene* 21:8981–8993. doi:10.1038/sj.onc.1206176