

Twenty Years of *BRCA1* and *BRCA2* Molecular Analysis at MMCI – Current Developments for the Classification of Variants

Dvacet let molekulární analýzy genů *BRCA1* a *BRCA2* v MOÚ – aktuální vývoj v klasifikaci nálezů

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Summary

Background: Deleterious mutations in the *BRCA1* and *BRCA2* genes account for a considerable proportion of dominantly inherited breast and ovarian cancer susceptibility. The laboratory interpretation has always been dependent on the information available at the time of the report conclusion. The aim of this study has been to review the results from the *BRCA* testing at Masaryk Memorial Cancer Institute (MMCI). **Patients and methods:** Patients with suspected hereditary predisposition to breast/ovarian cancer, belonging to 7,400 families, were referred by genetic counsellors for *BRCA1* and *BRCA2* mutation testing at the MMCI from 1999 to the beginning of 2018. Various methods have been used over 20 years of laboratory practice – starting with the Protein Truncation Test and Heteroduplex Analysis via the High Resolution Melting analysis and Sanger sequencing up to Next Generation Sequencing. **Results:** *BRCA1* and *BRCA2* mutation screening resulted in the identification of 1,021 families with a germline high-risk *BRCA1* mutation and 497 families carrying a high-risk *BRCA2* mutation, representing a mutation detection rate of 20.5%. A broad spectrum of unique mutations classified as pathogenic or likely pathogenic has been detected in both genes – 124 in the *BRCA1* and 123 in the *BRCA2* gene. Other sequence variants (96 unique variants in the *BRCA1* and 126 in the *BRCA2* gene) have been revised and classified as benign or likely benign. The other 82 unique variants remain classified as of uncertain significance mainly due to a lack of information for inclusion in other groups. All the results are summarised in the tables, including the reasons for their classification. **Conclusion:** The clinical classification of rare sequence variants identified in the high-risk breast cancer susceptibility genes *BRCA1* and *BRCA2* is essential for appropriate genetic counselling. Here we present an overview of *BRCA* mutation frequencies in our region and the retrospective evaluation and eventually reclassification of previously reported rare variants in light of recent findings.

Key words

breast cancer – ovarian cancer – *BRCA1* gene – *BRCA2* gene – germline mutation

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Souhrn

Východiska: Patogenní mutace v genech *BRCA1* a *BRCA2* jsou majoritní příčinou dědičné dominantní predispozice ke vzniku nádoru prsu a vaječníku. Interpretace molekulárně-genetických nálezů vždy závisí na dostupných informacích v době uzavření laboratorní zprávy. Cílem této studie byla revize klasifikace všech výsledků testování *BRCA* genů v Masarykově onkologickém ústavu (MOÚ). **Soubor pacientů a metody:** Pacienti ze 7 400 rodin s podezřením na dědičnou predispozici ke vzniku nádorů prsu a/nebo vaječníků byli v MOÚ vyšetřeni v období let 1999 až první poloviny 2018. Vyšetření genů *BRCA* bylo vždy indikováno klinickým genetikem. V průběhu 20 let laboratorní praxe byly použity různé metody – počínaje vyšetřením cíleným na detekci zkrácené délky proteinu a heteroduplexní analýzu přes vysokorozlišovací analýzu křivek tání a Sangerovo sekvenování až po masivní paralelní sekvenování. **Výsledky:** Mutační analýza vedla k odhalení dědičné predispozice k nádoru prsu/ovaria u 20,5 % rodin. Vysoce riziková zárodečná mutace byla detekována u 1 021 rodin v genu *BRCA1* a u 497 rodin v genu *BRCA2*. Bylo zachyceno široké spektrum patogenních a pravděpodobně patogenních unikátních mutací v obou genech – 124 různých mutací v genu *BRCA1* a 123 různých mutací v genu *BRCA2*. Jako benigní nebo pravděpodobně benigní bylo klasifikováno 96 unikátních variant v genu *BRCA1* a 126 variant v genu *BRCA2*. Zbývajících 82 vzácných unikátních variant zůstalo klasifikováno jako „nejasného významu“, především z důvodu ojedinělého výskytu a nedostatku podkladů pro jejich zařazení do ostatních skupin. Výsledky jsou shrnuty v tabulkách dle typu mutace/varianty vč. podkladů pro jejich klasifikaci. **Závěr:** Co nejpřesnější klinická klasifikace variant identifikovaných v *BRCA* genech má dopad na genetické poradenství a následnou klinickou péči. V této studii uvádíme přehled frekvencí *BRCA* mutací detekovaných v našem regionu, retrospektivní hodnocení a případné reklasifikaci u některých dříve reportovaných variant ve světle nedávných zjištění.

Klíčová slova

nádor prsu – nádor ovaria – gen *BRCA1* – gen *BRCA2* – zárodečné mutace

Introduction

Several breast and ovarian cancer susceptibility genes have been identified to date. The most important genes in the context of genetic counselling remain the *BRCA1* and *BRCA2*. The germline *BRCA1/2* heterozygote frequency in individuals of European non-Finnish descent – *BRCA1* mutation frequency 0.21% (1 : 480) and *BRCA2* mutation frequency 0.31% (1 : 327); both combined 0.51% (1 : 195) [1] was calculated by examining publicly available data from the Exome Variant Server and the Exome Aggregation Consortium database. However, these calculations do not incorporate large genomic rearrangements or uncharacterised, but potentially pathogenic, missense mutations and, therefore, could be underestimates of true population frequencies of *BRCA1/2* heterozygotes [1]. These figures are in agreement with those obtained in an unselected population cohort of 50,726 adults who underwent exome sequencing: 0.52% (n = 267) were found to be *BRCA1/2* mutation carriers [2].

Mutations in the *BRCA1* (MIM#113705) and *BRCA2* (MIM#600185) account for an autosomal dominant transmission of susceptibility to breast and ovarian cancers. *BRCA1/2* genes have been studied very well since their discovery in 1994 and 1995. The cumulative breast cancer risk up to the age of 80 was determined at 72% for *BRCA1* and 69% for

BRCA2 mutation carriers [3]. Breast cancer incidences increased rapidly in early adulthood until the age of 30 to 40 for *BRCA1* and until the age of 40 to 50 for *BRCA2* mutation carriers, then remained relatively constant throughout the remainder of the patient's lifetime. The cumulative ovarian cancer risk up to the age of 80 was determined at 44% for *BRCA1* and 17% for *BRCA2* mutation carriers [3].

Genetic counselling has become an integral part of *BRCA1/2* testing and helps patients in making informed decisions about undergoing testing. The results are used to plan optimal women's treatment or clinical management options, which involve a combination of early cancer screening, prophylactic surgery and other risk reduction strategies.

Genetic testing may detect changes that are clearly pathogenic, clearly neutral or variants of uncertain clinical significance (VUS). Such variants present a considerable challenge to the diagnostic laboratory and the receiving clinician in terms of interpretation. Adequate classification of rare sequence changes identified in the high-risk breast cancer susceptibility genes *BRCA1* and *BRCA2* is essential for appropriate genetic counselling of individuals carrying these variants.

The aim of this study has been to review the results from the *BRCA* testing at Masaryk Memorial Cancer Insti-

tute (MMCI) during the last 20 years of diagnostic practice. This will give a necessary overview of mutation frequencies in our region and the retrospective evaluation and eventually reclassification of previously reported rare variants in light of recent findings. The reporting of novel sequence variants included a clinical interpretation based on the best data available at the time of testing. Often, as subsequent studies were done, either within the same family in our region or others reported in literature, this clinical interpretation may need to be modified or changed. When the causal status of a sequence variant is indeterminate, follow-up activities may be useful to clarify this relationship and assist risk assessment.

A system of five classes of variants based on the degree of likelihood of pathogenicity is used as recommended [4] in agreement with the ACMG (American College of Medical Genetics) [5] / ENIGMA (Evidence-based Network for the Interpretation of Germline Mutant Alleles) criteria [6]. Each class is associated with specific recommendations for clinical management of at-risk relatives.

Materials and methods

Patients, controls, and criteria for testing

The patients were referred to the MMCI in Brno for genetic counselling by phy-

sicians from various specialisations or were sent for testing by other medical geneticists from various parts of the Czech Republic between 1999 and 2018. All the tested individuals provided a signed informed consent following appropriate genetic counselling. Genetic testing was offered to high-risk individuals meeting the recommended criteria for *BRCA* testing. In this study, “family” was defined by the practice of giving an index patient (proband) a separate family number if he/she did not already have family members registered in our laboratory. If another relative came, this person was included in the already registered family. The results of the testing from 1999–2018 include 7,400 high-risk families referred for genetic testing in the context of a presumed genetic predisposition for breast and/or ovarian cancer. The criteria for genetic testing have been revised and edited over 20 years of genetic practice and have been published elsewhere in the framework of the guidelines for the Czech Republic [7,8].

The control “cancer-free” group was composed of healthy individuals (150) above 60 years of age without the occurrence of cancer in their personal history and without the occurrence of tumours of the breast, ovaries, prostate or colon cancer in their first- or second-degree relatives. All the control individuals provided a signed informed consent with participation for the purposes of research.

Mutation screening

Genomic DNA was isolated from blood samples with a QIAamp DNA blood purification kit (Qiagen). Initially, individuals from approximately the first 1,000 families were analysed during 1999–2006 using the Protein Truncation Test and Heteroduplex Analysis followed by Sanger sequencing on the ALF express™ DNA sequencer (Pharmacia) described elsewhere [8].

High-Resolution Melting (HRM) curve analysis was used to analyse individuals from 5,900 families during 2007–2017. To cover the complete coding region and splice sites of *BRCA1* and *BRCA2*, 89 polymerase chain reaction (PCR) amplicons

(200–540 bp) were amplified. LCGreen® Plus was used as the intercalating dye for HRM analysis performed on a 96-well LightScanner™ (Idaho Technology Inc.). Later (starting in 2012), the analysis of several highly polymorphic amplicons was transferred to dHPLC (Denaturing High-Performance Liquid Chromatography; Transgenomic Wave system 4500) for better resolution of polymorphic regions. Sanger sequencing was performed on 3130 Genetic Analyser (Applied Biosystems) from 2007 with a BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

In addition, we evaluated the frequency of large genomic rearrangements in the *BRCA1* gene with Multiplex Ligation-dependent Probe Amplification (MLPA) (MRC-Holland). More details about our pilot study have been reported elsewhere [9]. MLPA for the *BRCA1* gene (SALSA MLPA Probemix P002; and confirmation Probemix P087; MRC Holland) has been the standard test since 2005. Focused on *BRCA2*, after testing 1,000 high-risk individuals without the detection of any exon-spanning deletion/duplication, MLPA for the *BRCA2* gene has been removed from the standard protocol. MLPA for *BRCA2* (SALSA MLPA Probemix P045; MRC Holland) was re-introduced as a standard test in 2015. Fragment analysis was performed on a 3130 Genetic Analyser (Applied Biosystems); free MLPA analysis software, Coffalyser.net was used for data analysis (MRC-Holland) together with a visual inspection of the fragment analysis profile.

Next generation sequencing methods were introduced in our laboratory in 2014 and several hundred individuals from 500 unrelated families referred for hereditary breast and/or ovarian cancer predisposition were tested up to mid-2018. Next generation sequencing was performed on a MiSeq system (Illumina).

We started with a commercially available targeted enrichment TruSight Cancer panel (Illumina) including 94 cancer predisposition genes along with the *BRCA1/2* genes. All the procedures were performed according to the manufacturers' instructions – Trusight-rapid-capture-sample-prep protocol (Illumina).

The first experiences with the TruSight Cancer panel have been published elsewhere [10]. Two hundred families were analysed with the TruSight cancer panel.

From 2016 onwards we have used the NimbleGen SeqCap EZ Choice (Roche) to create a sequencing library with a multi-gene panel called CZECA (CZEch CAncer paNel for Clinical Application) according to NimbleGen SeqCap EZ Library SR User's Guide [11]. Three hundred families were analysed with the CZECA cancer panel.

A FinalistDX integrated bioinformatics computing system (Institute of Applied Biotechnologies) with a Linux operating system (Ubuntu) was used for data processing and analysis of MiSeq FASTQ files. FinalistDX software allows fast and comprehensive bioinformatics analysis from raw FASTQ files to quality control, alignment to the reference genome (bam, bai), coverage analysis, variants calling (VCF files) and recently also copy number variations (CNV) analysis with detailed reports in a variety of formats (tsv, xls, pdf). The alternative bioinformatics analysis, which was used in parallel, has been described elsewhere [11].

Nomenclature and variant classification

All sequence variants have been named and are referred to in the text according to the nomenclature used by the Human Genome Variation Society recommendation guidelines [12], using the A of the ATG-translation initiation codon as nucleotide +1 [13]. Detected sequence alterations are described at the coding DNA reference sequence (cDNA) level according to the *BRCA1* most common human transcript (NM_007294.3) with the traditional numbering of exons 1–24 without the presence of exon 4, and according to the *BRCA2* (NM_000059.3) reference sequences.

The variants were assessed in Alamut® Visual software (Interactive Biosoftware) and other public databases (BIC, LOVD, UMD, ClinVar) to determine whether they were known in other populations. All variants were evaluated regarding pathogenicity following the recommended terminology for classification [4–6]:

Tab. 1. *BRCA1* nonsense and frame-shift mutations detected in Czech patients.

cDNA level (HGVS nomenclature)	Protein level	Number of families	NFE frequency (gnom)	LOVD-IARC class [4] // our class (if different or not specified)
c.55C>T	p.Gln19*	2	–	class 5, pathogenic
c.68_69del	p.Glu23Valfs*17	24	0.0087%	class 5, pathogenic
c.143del	p.Met48Serfs*2	1	–	class 5, pathogenic
c.160C>T	p.Gln54*	1	–	class 5, pathogenic
c.303T>G	p.Tyr101*	1	–	class 5, pathogenic
c.485_486del	p.Val162Glufs*19	1	–	class 5, pathogenic
c.505C>T	p.Gln169*	2	–	class 5, pathogenic
c.622_625dup	p.Pro209Hisfs*8	1	– // novel	– // class 4: likely pathogenic; putative hypomorphic allele?
c.676del	p.Cys226Valfs*8	1	–	class 5, pathogenic
c.737del	p.Leu246*	1	–	class 5, pathogenic
c.798_799del	p.Ser267Lysfs*19	3	–	class 5, pathogenic
c.843_846del	p.Ser282Tyrfs*15	7	–	class 5, pathogenic
c.890del	p.Met297Argfs*3	1	– // novel	– // class 5, pathogenic
c.962G>A	p.Trp321*	1	0.00090%	class 5, pathogenic
c.963G>A	p.Trp321*	1	–	class 5, pathogenic
c.1016dup	p.Val340Glyfs*6	7	–	class 5, pathogenic
c.1016del	p.Lys339Argfs*2	5	–	class 5, pathogenic
c.1040del	p.Leu347Argfs*27	1	–	class 5, pathogenic
c.1068_1077del	p.Gln356Hisfs*15	1	–	class 5, pathogenic
c.1127del	p.Asn376Ilefs*18	9	–	class 5, pathogenic
c.1204del	p.Glu402Serfs*8	4	–	class 5, pathogenic
c.1273dup	p.Ser425Phefs*11	5	–	class 5, pathogenic
c.1403del	p.Lys468Argfs*7	1	–	class 5, pathogenic
c.1416del	p.Asn473Thrfs*2	2	–	class 5, pathogenic
c.1504_1508del	p.Leu502Alafs*2	1	–	class 5, pathogenic
c.1510del	p.Arg504Valfs*28	7	–	class 5, pathogenic
c.1600C>T	p.Gln534*	5	–	class 5, pathogenic
c.1687C>T	p.Gln563*	36	0.0054%	class 5, pathogenic
c.1938_1947del	p.Ser646Argfs*2	1	–	class 5, pathogenic
c.1953_1956del	p.Lys653Serfs*47	1	–	class 5, pathogenic
c.2022_2031dup	p.Ala678Cysfs*8	1	– // novel	– // class 5, pathogenic
c.2024dup	p.Thr676Asnfs*7	2	– // novel	– // class 5, pathogenic
c.2070_2071del2	p.Arg691Thrfs*2	1	–	class 5, pathogenic
c.2157dup	p.Glu720Argfs*6	2	–	class 5, pathogenic
c.2193_2196del	p.Glu732Argfs*3	1	–	class 5, pathogenic
c.2233del	p.Glu745Lysfs*8	1	– // novel	– // class 5, pathogenic

cDNA – coding DNA reference sequence, HGVS – Human Genome Variation Society, NFE – non-Finnish European, LOVD – Leiden Open Variation Database, IARC – International Agency for Research on Cancer

Tab. 1 – continuing. *BRCA1* nonsense and frame-shift mutations detected in Czech patients.

cDNA level (HGVS nomenclature)	Protein level	Number of families	NFE frequency (gnom)	LOVD-IARC class [4] // our class (if different or not specified)
c.2263G>T	p.Glu755*	9	–	class 5, pathogenic
c.2371_2372del	p.Leu791Argfs*9	1	– // novel	– // class 5, pathogenic
c.2411_2412del	p.Gln804Leufs*5	15	–	class 5, pathogenic
c.2488_2497dup	p.Leu833*	15	–	class 5, pathogenic
c.2683C>T	p.Gln895*	1	–	class 5, pathogenic
c.2762del	p.Gln921Argfs*79	10	–	class 5, pathogenic
c.3018_3021del	p.His1006Glnfs*17	1	–	class 5, pathogenic
c.3164del	p.Gly1055Alafs*4	1	–	class 5, pathogenic
c.3239T>A	p.Leu1080*	2	–	class 5, pathogenic
c.3607C>T	p.Arg1203*	1	0.0018%	class 5, pathogenic
c.3621_3626delinsAA	p.Leu1209Serfs*25	1	–	class 5, pathogenic
c.3640G>T	p.Glu1214*	2	–	class 5, pathogenic
c.3642_3643del	p.Asn1215Leufs*3	10	–	class 5, pathogenic
c.3700_3704del	p.Val1234Glnfs*8	114	–	class 5, pathogenic
c.3756_3759del	p.Ser1253Argfs*10	16	0.0027%	class 5, pathogenic
c.3770_3771del	p.Glu1257Glyfs*9	3	0.00090%	class 5, pathogenic
c.3937C>T	p.Gln1313*	1	–	class 5, pathogenic
c.4035del	p.Glu1346Lysfs*20	2	0.0087%	class 5, pathogenic
c.4052T>A	p.Leu1351*	5	–	class 5, pathogenic
c.4062_4068del	p.Asn1354Lysfs*10	1	–	class 5, pathogenic
c.4065_4068del	p.Asn1355Lysfs*10	2	0.00090%	class 5, pathogenic
c.4161_4162del	p.Gln1388GluX2	1	–	class 5, pathogenic
c.4165_4166del	p.Ser1389*	1	0.00090%	class 5, pathogenic
c.4237G>T	p.Glu1413*	1	–	class 5, pathogenic
c.4243del	p.Glu1415Lysfs*4	3	0.00090%	class 5, pathogenic
c.4299_4302del	p.Ile1433Metfs*22	1	– // novel	– // class 5, pathogenic
c.4339C>T	p.Gln1447*	1	–	class 5, pathogenic
c.4524G>A	p.Trp1508*	1	–	class 5, pathogenic
c.4530dup	p.His1511Alafs*10	1	– // novel	– // class 5, pathogenic
c.4545del	p.Ser1516Valfs*32	1	– // novel	– // class 5, pathogenic
c.4666C>T	p.Gln1556*	1	–	class 5, pathogenic
c.4689C>G	p.Tyr1563*	6	0.0018%	class 5, pathogenic
c.4806del	p.Gln1604Asnfs*2	1	–	class 5, pathogenic
c.4878dup	p.Ala1627Cysfs*52	2	–	class 5, pathogenic
c.5030_5033del	p.Thr1677Ilefs*2	2	–	class 5, pathogenic
c.5177_5178del	p.Arg1726Lysfs*5	2	–	class 5, pathogenic
c.5251C>T	p.Arg1751*	13	0.00079%	class 5, pathogenic

cDNA – coding DNA reference sequence, HGVS – Human Genome Variation Society, NFE – non-Finnish European, LOVD – Leiden Open Variation Database, IARC – International Agency for Research on Cancer

Tab. 1 – continuing. *BRCA1* nonsense and frame-shift mutations detected in Czech patients.

cDNA level (HGVS nomenclature)	Protein level	Number of families	NFE frequency (gnom)	LOVD-IARC class [4] // our class (if different or not specified)
c.5266dup	p.Gln1756Profs*74	329	0.016%	class 5, pathogenic
c.5346G>A	p.Trp1782*	3	–	class 5, pathogenic
c.5432del	p.Gln1811Argfs*23	1	– // novel	– // class 5, pathogenic
c.5510G>A	p.Trp1837*	2	–	class 5, pathogenic
c.5511G>A	p.Trp1837*	2	–	class 5, pathogenic
c.5554del	p.Thr1852Profs*3	1	– // novel	– // class 5, pathogenic

cDNA – coding DNA reference sequence, HGVS – Human Genome Variation Society, NFE – non-Finnish European, LOVD – Leiden Open Variation Database, IARC – International Agency for Research on Cancer

- Class 5: pathogenic (probability of being pathogenic > 0.99);
- Class 4: likely pathogenic (probability of being pathogenic 0.95–0.99);
- Class 3: uncertain significance (probability of being pathogenic 0.05–0.949);
- Class 2: likely benign (probability of being pathogenic 0.001–0.049);
- Class 1: benign (probability of being pathogenic < 0.001).

Prediction of putative splice site variants and mRNA (cDNA) analysis

All putative splice site variants were tested using the Splice Site Prediction Programs for their potential to alter splicing. Several predictive programs were used as the NNSplice [14] or NetGene2 [15]. Later, Alamut® Visual (Interactive Biosoftware) predictions on ribonucleic acid (RNA) splicing, allowing the assessment of their potential impact on splice junctions and visualization of cryptic or *de novo* splice sites, was used. Splicing prediction tools included in Alamut are represented by NNSplice [14]; GeneSplicer [16]; MaxEntScan [17]; SpliceSiteFinder-like [18]. Subsequently, messenger RNA (mRNA) / cDNA analysis was performed to verify ‘*in silico*’ predictions in cases of previously not characterised putative splice variants as described elsewhere [8,19,20].

In silico analyses for missense and in-frame indel variants

Several prediction software were used including Grantham Variation (GV) and Grantham Deviation (GD) scores, later

Align-GVGD [21], which combines the biophysical characteristics (side-chain composition, polarity and volume) of amino acids and protein multiple sequence alignments. We also applied PRIORS V2.0. The *BRCA1* and *BRCA2* Prior Probabilities database combines Prior Probabilities of pathogenicity from missense substitution severity and spliceogenity [22].

Alamut® Visual software (Interactive Biosoftware) has been used since 2015, which integrates several missense variant pathogenicity prediction tools and algorithms such as SIFT, PolyPhen2, Align-GVGD or MutationTaster; as well as the splicing prediction tools mentioned above.

For in-frame deletions/insertions we used PROVEAN (Protein Variation Effect Analyser) software [23], which predicts whether an amino acid substitution or indel will have an impact on the biological function of a protein [24].

More recently, the free interactive database VarSome has been used as well, created by Saphetor SA for the human genomics annotation tool [25].

Definition of deleterious mutations

Sequence variants were categorised on the basis of their predicted effect on the mRNA and amino acid level and defined as deleterious mutations according to the ACMG/ENIGMA criteria [5,6]:

- 1) Frameshift and nonsense variants in both genes, with the exception of *BRCA2* variants leading to a stop codon 3’of codon 3326 as *BRCA2* c.9976A>T;

p.Lys3326* (rs11571833) has been found to be of clinically low significance, associated with only very slightly increased risk of breast cancer (OR_w 1.28; 95% CI 1.17–1.40, P = 5.9 × 10⁻⁶) [26]. Therefore, other variants leading to a stop codon 3’ of codon 3326 are considered as class 2 variants.

- 2) Variants occurring in the consensus splice acceptor or donor sequence sites, either within 2 bp of exon-intron junctions, when they are experimentally demonstrated to result in abnormal mRNA transcript and found to produce only transcript(s) carrying a premature termination codon, or an in-frame deletion disrupting the expression of one or more known clinically important residues.
- 3) Missense variants that have been conclusively demonstrated, on the basis of data from linkage analysis of high-risk families, functional assays or biochemical evidence, to have a deleterious effect on known functional domains.
- 4) Copy number deletion/duplication variant that removes/duplicates one or more exons spanning a known clinically important functional or is proven by laboratory studies to result in a frameshift alteration predicted to disrupt the expression of one or more known clinically important functional residues.

Results

***BRCA1* findings**

In total, there were 1,021 families confirmed to carry a high-risk *BRCA1* muta-

Tab. 2. BRCA1 splice site alterations detected in Czech patients.

cDNA level (HGVS nomenclature)	Number of families	In silico Prediction: Alamut average (MaxEnt/NNSPLICE/SSF)	[reference]: described mRNA analysis results // other important information	LOVD-IARC class [4] // our class (if different or not specified)
c.213-12A>G	33	new acceptor site: c.213-11; WT acceptor: -35.0%	[29]: r.212_213ins213-12_213-1; p.(Arg71Serfs*21)	class 5, pathogenic
c.213-11T>G	1	-96%	[52]: r.212_213ins213-59_213-1 UMD database: Intron inclusion: ins59Ter75	class 5, pathogenic
c.302-3C>G	1	new acceptor site: c.302-2; WT acceptor: -87,1%	[8]: r.302-2_302-1insAG; p.(Tyr101*)	class 5, pathogenic
c.4096+3A>T	1	-99.1%	[27]: enhanced Δ Ex 11; Δ 3309nt 3' of Ex 11: in-frame p.[(Ala224_Leu1365del);(Ser264_Leu1365del)] // [28]: healthy 58-years-old homozygous carrier of c.4096+3A>G	class 3, VUS
c.4185G>A	5	-68.9%	[19]: r.4097_4185del; p.(Gly1366Alafs*8)[57]: r.4097_4185del	class 5, pathogenic
c.4185+1G>C	1	-100%	novel; predicted exon skipping: r.(4097_4185del); several other pathogenic splice site alteration described all with exon skipping effect: c.4185+1G>T, c.4185+1G>A, c.4185+2T>C, c.4185G>A	class 4, likely pathogenic
c.4675+1G>A	1	-100%	[8]: several aberrant transcripts including minor wild-type and in-frame transcript: r.[=, 4358_4675del, 4485_4675del, 4665_4675del, 4358_4484del, 4665_4675del] p. [=, Ala1453_Leu1558del, Ser1496Glyfs*14, Gln1556Glyfs*14, Ala1453Glyfs*10][57]: r.4485_4675del; [58]: Ex 15 deletion, 11-bp deletion from Ex 15	class 4, likely pathogenic possible spliceogenic effect?
c.4674A>G; p.(Leu1558=)	1	-48.8%	[20]: r.4665_4675del; p.(Gln1556Glyfs*14)	class 5, pathogenic
c.4676-1G>A	11	-100%	// [59]: multifactorial likelihood ratio - pathogenic	class 5, pathogenic
c.4868C>G, p.(Ala1623Gly)	1	new donor site; prior score: 0,64	[60]: r.4868_4986del p.(Ala1623Aspfs*16) // [61]: affects function	class 5, pathogenic
c.5074+3A>G	2	-61.5%	[62]: Ex 17 skipping and retention of 153 nt of intron 17; LOVD database: Ex 17 skipping: r.4987_5074del => frame-shift	class 5, pathogenic
c.5075-1G>A	2	-100%	LOVD database: in-frame Ex 18 skipping r.5075_5152del => in-frame deletion p.(Asp1692_Trp1718delinsGly)	class 5, pathogenic
c.5152+1G>C	1	-100%	[61]: affects function: r.5075_5152del => in-frame deletion	class 5, pathogenic
c.5152+1G>T	1	-100%	[61]: affects function: r.5075_5152del => in-frame deletion	class 5, pathogenic
c.5152+2dupT	4	-93.2%	[20]: Ex 18 skipping r.5075_5152del => in-frame deletion p.(Asp1692_Trp1718delinsGly)[61]: affects function: r.5075_5152del => in-frame deletion	class 5, pathogenic
c.5468-1G>A	1	-100%, new acceptor splice site: c.5479	[20]: new acceptor site activated, deletion of first 11nt of Ex: r.5468_5478del => frame-shift p.(Ala1823Aspfs*3)	class 5, pathogenic
c.5468-11_5520dup64	5	? tandem duplication	[61]: affects function: r.?.; predicted frame-shift: p.(Ala1843Serfs*8)	class 4, likely pathogenic

cDNA – coding DNA reference sequence, HGVS – Human Genome Variation Society, mRNA – messenger RNA, LOVD – Leiden Open Variation Database, IARC – International Agency for Research on Cancer, WT – wild type, VUS – variants of uncertain clinical significance

Tab. 3. *BRCA1* missense variants detected in Czech patients classified as pathogenic (class-5) and likely pathogenic (class-4)

cDNA level (HGVS nomenclature)	Protein	Number of families	NFE freq. (gnom)	Prior	Align-GVGD	[reference]: functional defect	[reference]: other information	LOVD-IARC class [4] // our class (if different or not specified)
c.53T>A	p.Met18Lys	17	–	0,66	C55	[35]: disrupted helical bundle; affects E2 (UbcH5b) binding // [36]: destabilising <i>BRCA1</i> /BARD1 complex	segregation with BC and OV cases in multiple families [37] in our recent study	class 4, likely pathogenic
c.71G>A	p.Cys24Tyr	1	–	0,81	C65	[35]: loss of Zn ²⁺ ligand residue of C ₃ HC ₄ RING domain		class 4, likely pathogenic
c.80G>A	p.Cys27Tyr	1	– // novel	0,81	C65	[35]: loss of Zn ²⁺ ligand residue of C ₃ HC ₄ RING domain	splice prediction: –10,8%;	class 4, likely pathogenic
c.115T>C	p.Cys39Arg	21	–	0,81	C65	[35]: loss of Zn ²⁺ ligand residue of C ₃ HC ₄ RING domain	[63]: deleterious; [34]: pathogenic; [64]: pathogenic mutation	class 5: pathogenic
c.181T>G	p.Cys61Gly	69	0.0063%	0,81	C65	[35]: loss of Zn ²⁺ ligand residue of C ₃ HC ₄ RING domain	[63]: deleterious; [34]: pathogenic	class 5: pathogenic
c.191G>A	p.Cys64Tyr	2	–	0,81	C65	[35]: loss of Zn ²⁺ ligand residue of C ₃ HC ₄ RING domain	[61]: affects function; [64]: pathogenic mutation	class 5: pathogenic
c.5054C>A	p.Thr1685Asn	1	– // novel	0,66	C55	BRCT domain	[34]: other variants: Thr1685Ile and Thr1685Ala – reported pathogenic	– // class 4, likely pathogenic
c.5089T>C	p.Cys1697Arg	1	–	0,81	C65	BRCT domain; [65]: folding defect, compromised transcription assay, but uncertain binding specificity	[66]: loss of transactivation activity in yeast and mammalian cells	– // class 4, likely pathogenic
c.5095C>T	p.Arg1699Trp	1	0.0018%	0,81	C65	BRCT domain; [65]: strong functional effect	[34]: pathogenic; [64]: pathogenic mutation	class 5: pathogenic
c.5096G>A	p.Arg1699Gln	1	0.0054%	0,66	C35	BRCT domain; [65]: strong functional effect	[67]: mRNA: no change [34]: pathogenic; [39]: hypomorphic risk: OR 4,3 (95% CI 1.43–12.85)	hypomorphic risk: OR 4,3

cDNA – coding DNA reference sequence, HGVS – Human Genome Variation Society, GVGD – Grantham Variation and Grantham Deviation, NFE – non-Finnish European, LOVD – Leiden Open Variation Database, IARC – International Agency for Research on Cancer, BRCT – *BRCA1* C-terminal, OR – odds ratio, VUS – variants of uncertain clinical significance

Tab. 3 – continuing. *BRCA1* missense variants detected in Czech patients classified as pathogenic (class-5) and likely pathogenic (class-4)

cDNA level (HGVS nomenclature)	Protein	Number of families	NFE freq. (gnom)	Prior	Align-GVGD	[reference]: functional defect	[reference]: other information	LOVD-IARC class [4] // our class (if different or not specified)
c.5123C>A	p.Ala1708Val	1	0.0027%	0,81	C65	BRCT domain; [65]: strong functional effect	[30]: partial exon skipping; r.5075_5152del;	class 3: VUS // class 4: likely pathogenic
c.5145C>G	p.Ser1715Arg	1	–	0,81	C65	BRCT domain; [65]: strong functional effect; [66]: loss of transactivation activity in yeast and mammalian cells;	[34]: pathogenic; [64]: pathogenic mutation	class 5: pathogenic
c.5360G>A	p.Cys1787Tyr	1	– // novel	0,81	C65	BRCT domain	[68]: p.Cys1787Ser is classified as pathogenic	– // class 4, likely pathogenic

cDNA – coding DNA reference sequence, HGVS – Human Genome Variation Society, GVGD – Grantham Variation and Grantham Deviation, NFE – non-Finnish European, LOVD – Leiden Open Variation Database, IARC – International Agency for Research on Cancer, BRCT – *BRCA1* C-terminal, OR – odds ratio, VUS – variants of uncertain clinical significance

tion (13.8% of the 7,400 families analysed). The majority of clinically deleterious mutations detected in the *BRCA1* gene were protein-truncating mutations (731 families with frameshift or nonsense mutations), followed by missense mutations located in the RING domain and less frequently by BRCT (*BRCA1* C-terminal) domains (118 families), large intragenic rearrangements (102 families) and splice site mutations (70 families).

An overview of the *BRCA1* frameshift and nonsense mutations detected in Czech high-risk families is provided in Tab. 1. We identified 731 families with frameshift or nonsense mutations in the *BRCA1* gene, which accounted for 79 different unique deleterious mutations. The most common mutations are c.5266dup (p.Gln1756Profs*74) detected in 329 families; c.3700_3704del (p.Val1234Glnfs*8), detected in 114 families; c.1687C>T (p.Gln563*), detected in 36 families; c.68_69del (p.Glu23Valfs*17), detected in 24 families.

The *BRCA1* splice site mutations detected in high-risk Czech families are

summarised in Tab. 2. They concern 71 families, representing 16 different deleterious splice site mutations classified as pathogenic (class 5) or likely pathogenic (class 4). One splice site alteration c.4096+3A>T leading to 2 in-frame transcripts at the cDNA level (exon 11 skipping and Δ3309nt 3'end of exon 11) and previously classified as pathogenic by [27] was reclassified as a variant of VUS, based on segregation data and the finding of a healthy 58-year-old homozygous woman for c.4096+3A>G variant in a consanguineous Danish family with several cases of breast/ovarian cancer [28]. However, in the Danish population *BRCA1* c.4096+3A>G is now considered as likely benign [28]. The most common splice site mutation was c.213-12A>G (detected in 33 families), activating a cryptic splice site and causing a frameshift [29].

All *BRCA1* pathogenic missense mutations are shown in Tab. 3. In 118 families, 13 different unique missense alterations, classified as pathogenic (class 5) or likely pathogenic (class 4) were detected. The

most common missense mutations were in the RING domain affecting the Zn²⁺ ligand residues of C₃HC₄ finger domain – p.Cys61Gly (detected in 69 families) and p.Cys39Arg (detected in 21 families).

An overview of all large intragenic rearrangements detected in the *BRCA1* gene have been provided in Tab. 4. There were 102 families with large intragenic rearrangements, representing 16 different unique mutations classified as pathogenic (class 5) or likely pathogenic (class 4). The most common large intragenic rearrangements were deletions spanning exons 5_14 (c.135-485_4485-913del), detected in 36 families and spanning exons 21_22 (c.5278-492_5406+1290delins236), detected in 21 families.

A summary of *BRCA1* in-frame deletions/insertions detected in Czech high-risk families is provided in the supplementary Tab. 5. All of them are considered as VUS (class 3).

The remaining *BRCA1* missense alterations classified as VUS (class 3), likely benign (class 2) and benign (class 1)

Tab. 4. *BRCA1* large intragenic rearrangements detected in Czech patients.

<i>BRCA1</i> (NM_007294.3) exons affected	HGVS nomenclature	Number of families	[reference]: breakpoints; predicted change	LOVD-IARC class [4]) // our class (if different or not specified)
deletion of promoter 1a_2	c.(?-232)_(80+1_81-1)del	8	deletion detectable by primers published by [69]: deletion of 36.9 kb;	class 5, pathogenic
deletion of exons 1a_17	c.(?-232)_(5074+1_5075-1)del	10	breakpoints not characterized	class 4, likely pathogenic
deletion of exons 5_14	c.135-485_4485-913del Chr17(GRCh38):g.43075434_43107018del	36	[9]: breakpoints characterised on RefSeq L78833.1: g.21716_53298del	class 5, pathogenic
deletion of exon 8	c.(441+1_442-1)_(547+1_548-1)del	7	breakpoints not characterized; deletion size about 4 kb (LR-PCR); predicted: r.(442_547del) => frame-shift	class 5, pathogenic
deletion from exon 11_12	c.1644_4185+3618del Chr17(GRCh38):g.43087326_43093887del	2	[9]: breakpoints characterised on RefSeq L78833.1: g.34845_41405del; predicted: r.?	class 4, likely pathogenic
duplication of exon 13	c.(4185+1_4186-1)_(4357+1_4385-1)dup	2	breakpoints not characterized; predicted (if tandem): r.(4186_4357)dup => frame-shift	class 4, likely pathogenic
duplication of exons 13_14	c.(4185+1_4186-1)_(4484+1_4485-1)dup	2	breakpoints not characterized; predicted (if tandem): r.(4186_4484)dup => frame-shift	class 4, likely pathogenic
deletion of exons 13_15	c.(4185+1_4186-1)_(4675+1_4676-1)del	1	breakpoints not characterized; predicted: r.(4186_4675)del => frame-shift	class 5, pathogenic
deletion of exon 17	c.(4986-1_4987+1)_(5074+1_5075-1)del	1	breakpoints not characterized; deletion size about 2,4 kb (LR-PCR) predicted: r.(4987_5074)del => frame-shift	class 5, pathogenic
deletion of exons 18_19	c.5075-1131_5193+190del Chr17(GRCh38):g.43063143_43065082del	1	[9]: breakpoints characterised on GeneBank RefSeq L78833.1: g.63651_65590del; predicted: r.(5075_5193)del => frame-shift	class 5, pathogenic
deletion of exons 18_22	c.(5074-1_5075+1)_(5406+1_5407-1)del	3	breakpoints not characterized; predicted: r.(5075_5406)del => frame-shift	class 5, pathogenic
deletion of exon 20	c.5193+340_5277+2206del Chr17(GRCh38):-g.43054846_43062993del	1	breakpoints characterised on GeneBank RefSeq L78833.1 (our study): g.65740_73887del; predicted: r.(5194_5277)del => in-frame deletion p.(His1732_Lys1759del)	class 5, pathogenic
deletion of exon 20	c.5194-2834_5277+4111del Chr17(GRCh38):g.43052941_43059969del	1	[9]: breakpoints characterised on GeneBank RefSeq L78833.1: g.68764_75792del; predicted: r.(5194_5277del) => in-frame: p.(His1732_Lys1759del)	class 5, pathogenic
deletion of exons 21_22	c.5278-492_5406+1290delins236 Chr17(GRCh38):g.43047831_43051609delins236	21	[9]: breakpoints characterised on GeneBank RefSeq L78833.1: g.77128_80906delins236; predicted: r.(5278_5406)del => in-frame p.(Ile1760_Thr1802del)	class 5, pathogenic
duplication of exon.23	c.(5406+1_5407-1)_(5467+1_5468-1)dup	1	breakpoints not characterized; predicted (if tandem): r.(5407_5467)dup => frame-shift	class 4, likely pathogenic
deletion of the entire <i>BRCA1</i> gene (exons 1a_24)	c.(?-232)>(*1_?)del	5	breakpoints not characterized; the whole allele deleted	class 5, pathogenic

HGVS – Human Genome Variation Society, LOVD – Leiden Open Variation Database, IARC – International Agency for Research on Cancer, LR-PCR – long-range polymerase chain reaction

Tab. 8. BRCA2 nonsense and frame-shift mutations detected in Czech patients.

cDNA level (HGVS nomenclature)	Protein level	Number of families	NFE frequency (gnom)	LOVD-IARC class [4] // our class (if different or not specified)
c.-7_7del	p.?	1	- // novel	- // class 5, pathogenic
c.306dup	p.Leu103Ilefs*10	1	-	- // class 5, pathogenic
c.462_463del	p.Asp156*	4	-	class 5, pathogenic
c.593T>G	p.Leu198*	1	- // novel	- // class 5, pathogenic
c.635_636del	p.Arg212Lysfs*2	3	-	class 5, pathogenic
c.658_659del	p.Val220Ilefs*4	14	0.0048%	class 5, pathogenic
c.691_704del	p.Tyr232*	1	- // novel	- // class 5, pathogenic
c.755_758del	p.Asp252Valfs*24	1	0.0018%	class 5, pathogenic
c.1023_1024del	p.Cys341*	1	- // novel	- // class 5, pathogenic
c.1296_1297del	p.Asn433Glnfs*18	2	-	class 5, pathogenic
c.1310_1313del	p.Lys437Ilefs*22	1	-	class 5, pathogenic
c.1389_1390del	p.Val464Glyfs*3	6	-	class 5, pathogenic
c.1408dup	p.Glu470Glyfs*6	1	-	class 5, pathogenic
c.1414C>T	p.Gln472*	1	-	class 5, pathogenic
c.1560_1561del	p.Ser521Argfs*5	1	-	class 5, pathogenic
c.1796_1800del	p.Phe599*	3	0.00090%	class 5, pathogenic
c.1813dup	p.Ile605Asnfs*11	5	0.0019%	class 5, pathogenic
c.1989del	p.Phe663Leufs*5	3	-	class 5, pathogenic
c.2251dup	p.Thr751Asnfs*2	2	-	class 5, pathogenic
c.2808_2811del	p.Ala938Profs*21	26	0.0027%	class 5, pathogenic
c.2828_2831del	p.Ile943Lysfs*16	1	-	class 5, pathogenic
c.2905C>T	p.Gln969*	1	-	class 5, pathogenic
c.3075_3076delinsTT	p.Lys1025_Lys1026delinsAsn*	6	-	class 5, pathogenic
c.3085del	p.Met1029Cysfs*14	2	- // novel	- // class 5, pathogenic
c.3195_3198del	p.Asn1066Leufs*10	4	-	class 5, pathogenic
c.3545_3546del	p.Phe1182*	1	0.0045%	class 5, pathogenic
c.3641dup	p.Phe1216Valfs*2	1	-	class 5, pathogenic
c.3703C>T	p.Gln1235*	1	- // novel	- // class 5, pathogenic
c.3744_3747del	p.Ser1248Argfs*10	3	-	class 5, pathogenic
c.3847_3848del	p.Val1283Lysfs*2	16	0.0082%	class 5, pathogenic
c.3860del	p.Asn1287Ilefs*6	1	0.0022%	class 5, pathogenic
c.3967A>T	p.Lys1323*	1	-	class 5, pathogenic
c.3975_3978dup	p.Ala1327Cysfs*4	4	0.00100%	class 5, pathogenic
c.4284dup	p.Gln1429Serfs*9	3	0.0067%	class 5, pathogenic
c.4440T>G	p.Tyr1480*	7	-	class 5, pathogenic
c.4544delA	p.Lys1515Argfs*28	4	-	class 5, pathogenic
c.4558_4559insT	p.Thr1520Ilefs*9	1	- // novel	- // class 5, pathogenic
c.4829_4830del	p.Val1610Glyfs*4	1	0.00090%	class 5, pathogenic
c.4845_4846del	p.Leu1616Lysfs*2	1	-	class 5, pathogenic
c.4987_4990del	p.Val1663Leufs*6	1	-	class 5, pathogenic
c.5042_5043del	p.Val1681Glufs*7	1	-	class 5, pathogenic
c.5073dup	p.Trp1692Metfs*3	1	0.0028%	class 5, pathogenic
c.5101C>T	p.Gln1701*	1	-	class 5, pathogenic
c.5213_5216del	p.Thr1738Ilefs*2	3	0.00090%	class 5, pathogenic
c.5216dup	p.Tyr1739*	1	-	class 5, pathogenic
c.5557dup	p.Cys1853Leufs*5	1	-	class 5, pathogenic
c.5576_5579del	p.Ile1859LysfsX3	2	0.0018%	class 5, pathogenic
c.5641_5644del	p.Lys1881Glnfs*27	10	-	class 5, pathogenic
c.5645C>A	p.Ser1882*	16	0.0018%	class 5, pathogenic
c.5682C>G	p.Tyr1894*	5	0.00090%	class 5, pathogenic
c.5720_5723del	p.Ser1907*	1	-	class 5, pathogenic
c.5722_5723del	p.Leu1908Argfs*2	1	-	class 5, pathogenic
c.5763dup	p.Ala1922Cysfs*2	2	-	class 5, pathogenic
c.5789del	p.Leu1930Tyrfs*33	1	-	class 5, pathogenic
c.5851_5854del	p.Ser1951Trpfs*11	1	-	class 5, pathogenic
c.5909C>G	p.Ser1970*	4	-	class 5, pathogenic

cDNA – coding DNA reference sequence, HGVS – Human Genome Variation Society, NC – not counted, NFE – non-Finnish European, LOVD – Leiden Open Variation Database, IARC – International Agency for Research on Cancer, mRNA – messenger RNA

are summarised in the supplementary Tab. 6, including the reasons for their classification. Silent variants were not included if, on the basis of the prediction the chance of affecting splicing was very low. A summary of *BRCA1* intronic variants targeted mainly up to position +/-50 to exon is provided in the supplementary Tab. 7.

BRCA2 findings

In total, there were 497 families confirmed to carry high-risk *BRCA2* mutation (6.7% of the 7,400 families analysed). Most clinically deleterious mutations detected in the *BRCA2* gene were protein-truncating mutations (404 families with deleterious frameshift or nonsense mutation), followed by splice site mutations (45 families), missense mutations with the exception of the mutation in the initiation codon located in the DBD (DNA/DSS1 binding) domain (44 families) and large intragenic rearrangements turned out to be very rare (4 families).

A summary of the frameshift and nonsense mutations detected in Czech high-risk families in *BRCA2* is provided in Tab. 8. In total, 404 families were identified with a truncating mutation, representing 95 different deleterious mutations. The most common mutations were: c.8537_8538del (p.Glu2846Glyfs*22), detected in 61 families; c.7913_7917del (p.Phe2638*), detected in 37 families; c.2808_2811del (p.Ala938Profs*21), detected in 26 families. Two variants leading to a stop codon 3' of codon 3326 were very frequent – p.Lys3326* and c.10095delC insGAATTATCT (p.Ser3366Asnfs*4).

An overview of the *BRCA2* splice site mutations detected in high-risk Czech families is shown in Tab. 9. There were 46 families carrying a splice site alteration, representing 9 different deleterious splice site mutations classified as pathogenic (class 5) or likely pathogenic (class 4). One splice site alteration, c.9501+3A>T, previously described as pathogenic and causing partial exon skipping (the aberrant transcript represented only 13% of the wild type transcript [30]) was reclassified as a variant of VUS. The most common splice site muta-

tion was c.475G>A, a substitution affecting the last nucleotide of exon 5 for which RNA analysis has demonstrated that it causes abnormal splicing and results in a frameshift and a truncated protein [8], was detected in 10 families.

The deleterious *BRCA2* missense mutations detected in high-risk Czech families can be found in Tab. 10. There were 44 families carrying a deleterious missense mutation, representing 15 different mutations classified as pathogenic (class 5) or likely pathogenic (class 4). With the exception of one mutation in the initiation codon, all deleterious missense mutations were located in the DBD domain. The most common missense mutation, classified as likely pathogenic was c.9371A>T (p.Asn3124Ile), detected in 16 families.

Of the total study cohort, only about 3,000 families were analysed for large intragenic rearrangements by MLPA (or later CNV analysis). Only 4 families were found to carry a large intragenic rearrangement in *BRCA2* (Tab. 11). In one of the complex *BRCA2* rearrangements we have not been able to reveal the exact character of the complex changes (Fig. 1). Because of the low frequency of *BRCA2* large intragenic rearrangements, we did not perform a retrospective analysis of all previously unsolved cases.

The remaining *BRCA2* missense alterations, classified as VUS (class 3), likely benign (class 2) and benign (class 1) are summarised in the supplementary Tab. 12, including the reasons for their classification. Silent variants were not included if, on the basis of the prediction the chance of affecting splicing was very low. A summary of the *BRCA2* intronic variants is provided in the supplementary Tab. 13.

During these 20 years the majority of variants, which were originally reported as of VUS, could be reclassified as likely benign or benign. As soon as information confirming the neutrality of any missense or intronic variant was available, we ceased to register their frequency and to report them. The frequency of neutral variants was not monitored because homozygotes of frequent variants were not detectable by screening with Heteroduplex nor HRM

analysis and therefore, their frequency would be underestimated.

Discussion

Deleterious mutations in the *BRCA1* and *BRCA2* genes account for a considerable proportion of dominantly inherited breast and ovarian cancer susceptibility and have received wide acceptance in diagnostic testing and prevention. The classification of sequence variants into high-risk or low-risk categories is both challenging and critical for clarification of the causative status. Only class 5 (pathogenic) and class 4 (likely pathogenic) can be used for guidance of clinical management. However, this classification system does not allow distinguishing between highly penetrant and hypomorphic alleles (like *BRCA1* c.5096G>A; p.Arg1699Gln) [31].

BRCA1 and *BRCA2* mutation screening in our laboratory resulted in the identification of 1021 families with deleterious *BRCA1* mutations and 497 families with a *BRCA2* mutation, resulting in a mutation detection ratio of 20.5%. A broad spectrum of different deleterious mutations was found in both genes – 124 unique mutations in *BRCA1* and 123 unique mutations in *BRCA2*. The most frequent were small frameshift and nonsense mutations – 174 unique mutations (79 in *BRCA1* and 95 in *BRCA2*) scattered in all regions of the coding sequences.

A missense mutation that leads to a non-conservative substitution of an evolutionarily conserved amino acid is more likely to be causative than a missense mutation that leads to a conservative substitution or alters an amino acid that is not evolutionarily conserved [5]. The PRIORS probability tool seems to have the strongest predictive value in agreement with already known deleterious missense mutations. The extent to which a sequence variation is considered causative of disease may be influenced by multiple parameters such as family history, segregation of the variant with affected relatives in a family, nature, and position of the amino acid substitution, evolutionary conservation of the affected residue, co-occurrence with a deleterious mutation, epidemiological and case/control studies, functional *in vitro*

Tab. 8 – continuing. *BRCA2* nonsense and frame-shift mutations detected in Czech patients.

cDNA level (HGVS nomenclature)	Protein level	Number of families	NFE frequency (gnom)	LOVD-IARC class [4] // our class (if different or not specified)
c.5934dup	p.Ser1979*	1	–	class 5, pathogenic
c.5946del	p.Ser1982Argfs*22	1	0.0079%	class 5, pathogenic
c.6275_6276del	p.Leu2092Profs*7	1	0.0048%	class 5, pathogenic
c.6295A>T	p.Arg2099*	2	– // novel	– // class 5, pathogenic
c.6405_6409del	p.Asn2135Lysfs*3	6	–	class 5, pathogenic
c.6444dup	p.Ile2149Tyrfs*2	6	–	class 5, pathogenic
c.6447_6448dup	p.Lys2150Ilefs*19	3	–	class 5, pathogenic
c.6449_6450del	p.Lys2150Serfs*25	3	–	class 5, pathogenic
c.6468_6469del	p.Gln2157Ilefs*18	4	–	class 5, pathogenic
c.6469C>T	p.Gln2157*	1	–	class 5, pathogenic
c.6486_6489del	p.Lys2162Asnfs*5	2	–	class 5, pathogenic
c.6553del	p.Ala2185Leufs*6	1	–	class 5, pathogenic
c.6591_6592del	p.Glu2198Asnfs*4	2	–	class 5, pathogenic
c.6638del	p.Ser2213Leufs*16	5	–	class 5, pathogenic
c.6754dup	p.Ser2252Phefs*9	5	–	class 5, pathogenic
c.6998dup	p.Pro2334Thrfs*6	1	0.00090%	class 5, pathogenic
c.7033C>T	p.Gln2345*	1	–	class 5, pathogenic
c.7069_7070del	p.Leu2357Valfs*2	1	0.0055%	class 5, pathogenic
c.7151_7152del	p.Gln2384Argfs*7	1	–	class 5, pathogenic
c.7471C>T	p.Gln2491*	3	–	class 5, pathogenic
c.7649del	p.Ile2550Lysfs*98	1	– // novel	– // class 5, pathogenic
c.7718T>A	p.Leu2573*	1	– // novel	– // class 5, pathogenic
c.7887G>A	p.Trp2629*	1	–	class 5, pathogenic
c.7913_7917del	p.Phe2638*	37	–	class 5, pathogenic
c.8042_8043del	p.Thr2681Serfs*11	8	–	class 5, pathogenic
c.8172_8175dup	p.Tyr2726Valfs*5	4	–	class 5, pathogenic
c.8322dup	p.Met2775Tyrfs*7	1	–	class 5, pathogenic
c.8343del	p.Asn2781Lysfs*40	1	–	class 5, pathogenic
c.8363G>A	p.Trp2788*	13	–	class 5, pathogenic
c.8537_8538del	p.Glu2846Glyfs*22	61	0.0027%	class 5, pathogenic
c.9053_9057del	p.Ser3018Ilefs*2	2	–	class 5, pathogenic
c.9097dup	p.Thr3033Asnfs*11	8	0.0028%	class 5, pathogenic
c.9145dup	p.Tyr3049Leufs*23	1	– // novel	– // class 5, pathogenic
c.9147C>A	p.Tyr3049*	1	–	class 5, pathogenic
c.9366_9367del	p.Ser3123Glnfs*26	1	–	class 5, pathogenic
c.9382C>T	p.Arg3128*	4	0.00079%	class 5, pathogenic
c.9403del	p.Leu3135Phefs*28	13	–	class 5, pathogenic
c.9435_9436del	p.Ser3147Cysfs*2	6	0.00090%	class 5, pathogenic
c.9463_9467delinsGAATGATC	p.Phe3155Glufs*2	2	–	class 5, pathogenic
c.9976A>T	p.Lys3326*	NC (> 40)	0.86%	class 1, benign; [76]: functional assays: no effect; [34]: benign; [26]: BC risk OR 1.28, (95% CI 1.17–1.40), OC risk OR 1.26 (95% CI 1.10–1.43); § (our study): detected in cancer free controls:
c.10095delC insGAATTATATCT	p.Ser3366Asnfs*4	NC (> 30)	–	class 2, likely benign; Ω [67]: mRNA analysis: no change; § (our study): detected in cancer free controls

cDNA – complementary DNA, HGVS – Human Genome Variation Society, NC – not counted, NFE – ?, LOVD – Leiden Open Variation Database, IARC – International Agency for Research on Cancer, mRNA – messenger RNA

studies or knock-out animal models [5]. A novel variant of VUS with neutral predictions in less conserved regions that are unlikely to affect splicing were always mentioned in the report but interpreted as a variant suspected to be of low clinical significance.

For *BRCA1* only missense variants located in the highly conserved RING finger domain at the N-terminal region (amino acids 1–109) and in the transcriptional activation domain with two BRCT repeats (amino acids 1640–1729 and 1760–1821) at the C-terminal region are considered

high-risk deleterious mutations [32], (LOVD database). In the *BRCA2* gene only some missense variants located in the most conserved C-terminal DNA binding DBD domain (amino acids 2460–3170) have been confirmed to be high-risk deleterious mutations [33,34], (LOVD database).

Tab. 9. *BRCA2* splice site alterations detected in Czech patients.

cDNA level (HGVS nomenclature)	Number of families	<i>In silico</i> Prediction: Alamut average (MaxEnt/NNSPLICE/SSF)	[reference]: described mRNA analysis // other important information	LOVD-IARC class [4] // our class (if different or not specified)
c.475G>A	10	-50.6%	[8]: r.426_475del p.(Phe143Glyfs*23)	class 5, pathogenic
c.475+1G>T	3	-100%	(our study): r.426_475del p.(Phe-143Glyfs*23); [52]: Ex 5 skipping r.426_475del	class 5, pathogenic
c.476-2A>G	6	-100%	[8]: two transcripts r.[426_516del, 476_516del] p.[Phe143Valfs*12; Val-159Glyfs*10]; [52] Colombo 2013: r.[=,476_516del,426_516del]	class 5, pathogenic // possible spliceogenic effect?
c.7007G>Ap. (Arg2336His)	9	-53.8%	[8]: three transcripts r.[6842_6937del, 6842_7007del, 6938_7007del] p.[Glu2281_Gly2313del, Gly2280Alafs*31, Gly2313Alafs*31]; [30]: Ex13 skipping: r.6938_7007del; [77]: ES cell-based assay: deleterious	class 5, pathogenic // possible spliceogenic effect?
c.7805+1G>C	1	-100%	Novel; recently described only c.7805+1G>A and c.7805+1G>T – both as pathogenic: r.spl (mRNA not characterised; LOVD database)	- // class 4, likely pathogenic
c.8486A>G p.(Gln2829Arg)	3	-11.8%	[78]: r.8332_8487del p.(Ile2778_Gln-2829del); [61]: in-frame deletion of Ex19; (our study): Ex 19 skipping in the main effect, but several alternative transcripts were observed also in control persons (skip of Ex 17,18, 20)	- // class 4, likely pathogenic; possible spliceogenic effect?
c.8755-1G>A	6	-100%	[8]: r.8755_8953del p.(Gly2919fs*3); [52] r.[=,8755_8953del, 8755_9004del] p. [Gly2919Leufs*3,Gly2919Lysfs*26]; [30]: cryptic splice (83%) and Ex 22 skipping (17%): r.[8755del,8755__8953del] p.[Gly1919Valfs*8,Gly2919Leufs*3]	class 5, pathogenic
c.9117+2T>A	4	-100%	[8]: r.8954_9117del p.(Val2985Glyfs*4)	class 5, pathogenic
c.9118-2A>G	3	-100%, strong cryptic acceptor	[19]: cryptic splice r.9118_9124del p.(Val3040Metfs*20)	class 5, pathogenic
c.9501+3A>T	1	-58%	[73]: incomplete Ex 25 skipping r.[=,9257_9501del] p.[=,Gly3086Glyfs*3] [58]: Ex 25 skipping: r.9257_9501del [49]: Partial Ex 25 skipping (13%): r.[=,9257_9501del]	conflicting: class 5, class 3 // class 3, VUS; strong spliceogenic effect, hypomorphic?

cDNA – coding DNA reference sequence, HGVS – Human Genome Variation Society, mRNA – messenger RNA, LOVD – Leiden Open Variation Database, IARC – International Agency for Research on Cancer, VUS – variants of uncertain clinical significance

Besides the five deleterious missense mutations at the strongly conserved C₃HC₄ Zn²⁺ ligand cysteine residues of the RING domain (Cys24, Cys27, Cys39, Cys61, Cys64), only p.Met18Lys, disrupting the helical bundle of the RING domain, affecting E2 (UbcH5b) binding and destabilising the

heterodimerisation of *BRCA1*/*BARD1* complex, was considered as a likely pathogenic variant [35–37]. *BRCA1* p.Met18Lys is a Czech founder mutation detected in 17 unrelated Czech families, with multiple breast and/or ovarian cancer patients, but is rare in other populations (ClinVar, LOVD

databases). More frequently p.Met18Thr has been reported, which is also classified as likely pathogenic [38] even with milder Align-GVGD predictions (C45 vs. C55 for p.Met18Lys).

Several previously classified deleterious missense mutations have been

found in the BRCT domains of the *BRCA1* gene: p.Arg1699Trp, p.Ser1715Arg, classified as definitively pathogenic (class 5); p.Cys1697Arg, p.Ala1708Val classified as likely pathogenic (class 4) on the basis of reported functional tests and strong 'in silico' predictions (Tab. 3). Two novel variants, p.Thr1685Asn, p.Cys1787Tyr were classified as likely pathogenic (class 4), because they are altering highly conserved residues, p.Thr1685 and p.Cys1787 with the same strong 'in silico' predictions as for both previously reported definitively pathogenic variants (p.Thr1685Ile, p.Thr1685Ala and p.Cys1787Ser, respectively) [34,38]. However, the penetrance of some missense variants may be lower, as a hypomorphic effect was shown for *BRCA1* p.Arg1699Gln, demonstrating ambiguous functional deficiency across multiple assays and calculated to be associated with reduced penetrance with estimated cumulative risks to age 70 of breast or ovarian cancer of 24% [39,31].

Besides *BRCA2* c.3G>A (p.Met1?) disrupting the translation initiation codon and classified as likely pathogenic (class 4) using a multifactorial analysis approach [40], several known deleterious missense mutations were detected in the conserved DBD domain of the *BRCA2* gene (Tab. 10): p.Trp2626Cys, p.Ile2627Phe, p.Asp2723His, p.Asp2723Gly, p.Arg3052Trp were classified as definitively pathogenic (class 5; LOVD, Enigma Rules); p.Gly2596Glu, p.His2623Arg, p.Lys2630Gln, p.Ser2670Leu, p.Arg2784Trp, p.Glu3002Lys, p.Gly3076Arg, p.Asn3124Ile were classified as likely pathogenic (class 4) on the basis of reported functional tests and strong 'in silico' predictions (Tab. 10). The novel *BRCA2* p.Glu2663Gly was classified as likely pathogenic (class 4) because it alters a highly conserved residue, p.Glu2663, with the same strong 'in silico' predictions as for previously reported definitively pathogenic variants altering p.Glu2663Val [34]. As for *BRCA1*, some of the *BRCA2* missense alterations might exert a hypomorphic effect. *BRCA2* p.Tyr3035Ser is associated with only a moderate risk of breast cancer – (OR 2.52; P = 0.04), similar to CHEK2 inactivating mutations [39]. In our family-1338 the p.Tyr3035Ser variant was detected in a woman also carrying the

BRCA2 nonsense mutation p.Ser1882* 'in trans'. This patient was diagnosed with bilateral breast cancer at the ages of 36 and 42 without symptoms of Fanconi anaemia.

Some variants were found in the nuclear localisation signals of *BRCA1* (amino acids 503–508; 606–615) [41]: p.Arg504His; p.Lys608del; or in the nuclear export sequences (amino acids 22–39 and amino acids 81–99) [32]: p.Pro25Leu; p.Lys38Arg – all of them classified as VUS (class 3). No variant was found in the nuclear localisation signals of *BRCA2* (NLS1 spanning amino acids 3263–3269; NLS2 spanning amino acids 3311–3317 and putative NLS3 spanning residues 3381–3385, which was found irrelevant) [42]. Thus, the most carboxy-terminal deleterious mutation in *BRCA2* was c.9463_9467delinsGAATGATC p.(Phe3155Glufs*2) removing both essential NLSs.

Some variants were found which affected the numerous phosphorylation sites in the *BRCA1* gene: p.Ser616del, Ser1542Cys and in *BRCA2* p.Pro3292Leu classified as VUS (class 3). *BRCA1* p.Ser1542Cys has been shown to eliminate ATM kinase binding, which is necessary for the phosphorylation of *BRCA1* in response to double-stranded breaks induced by γ irradiation [43]. *BRCA2* p.Pro3292Leu altered the sequence for CDK2 binding for Ser3291, and completely abolished kinase binding. It was shown previously that phosphorylation of Ser3291 by CDKs blocks the interaction between *BRCA2* and RAD51, serving as a molecular switch for the regulation of recombination activity, which suggests potential significance by negatively affecting the interaction between *BRCA2* and RAD51 [43].

All VUS variants summarised in our study may not have been detected in patients analysed with Heteroduplex Analysis and Protein Truncation Test at the beginning and later with High Resolution Melting analysis when the analysis was terminated after the detection of pathogenic mutation. Therefore, the presence of some VUS in our analysed group may be underestimated. Only patients without detected pathogenic mutations or with a double-sided risk were completely analysed even if

the high-risk mutation (class 5, definitively pathogenic) was detected. In two families with double-sided risk of breast and ovarian cancers a case was found with double *BRCA1* and *BRCA2* risk mutation – (family-340: *BRCA1* p.Met18Lys together with *BRCA2* p.Arg3052Trp – in a woman diagnosed with breast cancer at the age of 41 and with Fallopian tube cancer at age of 52) and (family-5597: *BRCA1* p.Met18Lys together with splice *BRCA2* c.7007G>A – in woman diagnosed with triple negative breast cancer at the age of 40).

The case report about one of our families with a double-sided risk of breast cancer and three affected children diagnosed with Fanconi anaemia carrying bi-allelic FANCD1/*BRCA2* mutations: c.[(658_659del);(9366_9367del)] has been described elsewhere [44]. The breast cancer genes, *BRCA1* and *BRCA2*, are both essential for viability, therefore at least one of these bi-allelic mutations in *BRCA2* in patients with Fanconi anaemia-D1 subtype should have a hypomorphic nature. *BRCA2* c.658_659del is described repeatedly in association with eight cases of Fanconi anaemia-D1 patients (LOVD database: Variants associated with Fanconi anaemia) whereas c.9366_9367del is submitted only once there for association with FA (our case; [44]). Therefore, the strongest candidate for *BRCA2* hypomorphic mutation is *BRCA2* c.658_659del (previously 886delGT by BIC nomenclature) in exon 8 described in association with Fanconi anaemia and medulloblastoma in several cases [44–46]. Several naturally occurring alternative transcripts in *BRCA2* were described [47] which might bypass the lethality of deleterious mutation by residual alternative splicing in some regions [48], however no natural alternative transcript interfering with exon 8 of *BRCA2* was described.

The first hypomorphic missense mutations with reduced penetrance were recently described with moderately increased risks of breast cancer among Europeans [39]. A similar effect is obvious for the spliceogenic variant that retains the ability to produce in-frame isoforms or residual full-length transcript [49–51], which has been repeatedly found as Fan-

Tab. 10. *BRCA2* missense variants detected in Czech patients classified as pathogenic (class 5) and likely pathogenic (class 4).

cDNA level (HGVS nomenclature)	Protein	Number of families	NFE freq. gnom	Align-GVGD	Prior	[reference]: functional defect // Ω mRNA analysis	[reference]: other information	LOVD-IARC class [4] // our class (if different or not specified)
c.3G>A	p.Met1?	2	–	0,96	–	initiation codon // Ω [40]: no splice aberration		class 4, likely pathogenic
c.7787G>A	p.Gly2596Glu	2	–	0,81	C65	[33]: HDR assay pathogenic		class 4, likely pathogenic
c.7868A>G	p.His2623Arg	1	–	0,29	C25	[33]: HDR assay pathogenic	[79]: protein likelihood ratio: likely deleterious,	class 4, likely pathogenic
c.7878G>C	p.Trp2626Cys	4	0.00090%	0,81	C65	[33]: HDR assay pathogenic	[79]: protein likelihood ratio: likely deleterious; [34]: pathogenic;	class 5, pathogenic
c.7879A>T	p.Ile2627Phe	1	–	0,29	C15	[76]: functional assays: inactivated; [80]: HDR assay: as mutant control; [33]: HDR assay pathogenic	[79]: protein likelihood ratio: likely deleterious; [34]: pathogenic;	class 5, pathogenic
c.7888A>C	p.Lys2630Gln	1	– // novel	0,66	C45	new donor site? [33]: HDR assay pathogenic		class 4, likely pathogenic
c.7988A>G	p.Glu2663Gly	5	– // novel	0,81	C65	functionally significant amino acid	[79]: protein likelihood ratio: E2663K and E2663V: likely deleterious; [34]: E2663V - pathogenic	– // class 4, likely pathogenic
c.8009C>T	p.Ser2670Leu	1	–	0,29	C15	[33]: HDR assay pathogenic // Ω [50]: Partial Ex 18 skipping (23%) r.[8009c>u;7977_8331del];	[79]: protein likelihood ratio: likely deleterious;	conflicting: class 3; class 4, // class 4, likely pathogenic
c.8167G>C	p.Asp2723His	2	–	0,81	C65	[76]: functional assays: inactivated; [80],[33]: HDR assay: pathogenic	[79]: protein likelihood ratio: likely deleterious; [34]: pathogenic;	class 5, pathogenic

cDNA – coding DNA reference sequence, HGVS – Human Genome Variation Society, NFE – non-Finnish European, mRNA – messenger RNA, LOVD – Leiden Open Variation Database, IARC – International Agency for Research on Cancer, HDR – homology directed repair, mouse ES – embryonic stem cells

coni anaemia-D1 associated *BRCA2* mutations. A possible reduced risk of spliceogenic and hypomorphic variants should be taken into consideration for clinical follow-up. Possible spliceogenic splice site alterations in the *BRCA2* gene detected in our study are marked in Tab. 9.

Residual full-length transcript was detected in *BRCA2* c.476-2A>G and c.8755-1G>A classified as pathogenic [52], whereas in c.9501+3A>T the full-length transcript was predominant and therefore the role in breast cancer risk is questionable and variant c.9501+3A>T

was reclassified as VUS [49]. Several *BRCA2* splice variants retain the ability to produce partially in-frame transcripts, which might have a residual function: c.7007G>A and c.8486A>G.

Spliceogenic variants are more frequent in *BRCA2*, but naturally occurring

Tab. 10 – continuing. *BRCA2* missense variants detected in Czech patients classified as pathogenic (class 5) and likely pathogenic (class 4).

cDNA level (HGVS nomenclature)	Protein	Number of families	NFE freq. gnom	Align-GVGD	Prior	[reference]: functional defect // Ω mRNA analysis	[reference]: other information	LOVD-IARC class [4] // our class (if different or not specified)
c.8168A>G	p.Asp2723Gly	3	–	0,81	C65	[76]: functional assays: inactivated; [80],[33]: HDR assay: pathogenic // Predicted new donor site Ω [67]; [72]: full length transcript + del of 164 nt from 3' of Ex 18. [50]: full length transcript (69,6%), Ex 18 del-164 (25,9%); ex 18 skipping (4,5%);	[79]: protein likelihood ratio: likely deleterious; [34]: pathogenic; [39]: pathogenic	class 5, pathogenic
c.8350C>T	p.Arg2784Trp	1	0.00090%	0,81	C65	[81]: functional complementation of mouse embryonic stem cells: poor survival, HDR capacity 15%; [33]: HDR assay: pathogenic	[79]: protein likelihood ratio: likely deleterious;	conflicting: class 3; class 4 // class 4, likely pathogenic
c.9004G>A	p.Glu3002Lys	1	–	0,66	C55	[82]: mouse ES cell-based assay: pathogenic; [33]: HDR assay: pathogenic; [81]: Functional complementation of mouse embryonic stem cells: inability to rescue the cell lethality	[79]: protein likelihood ratio: likely deleterious;	class 4, likely pathogenic
c.9154C>T	p.Arg3052Trp	3	0.00090%	0,81	C65	[76]: functional assays: inactivated; [80]: HDR assay: as mutant control // Ω [60]: no aberrant	[79]: protein likelihood ratio: likely deleterious; [34]: pathogenic; [39]: pathogenic	class 5, pathogenic
c.9226G>A	p.Gly3076Arg	1	– // novel	0,81	C65	[33]: HDR assay: pathogenic		– // class 4, likely pathogenic
c.9371A>T	p.Asn3124Ile	16	0.0018%	0,81	C65	[82]: mouse ES cell-based assay: pathogenic; [80],[33]: HDR assay: pathogenic	[79]: protein likelihood ratio: likely deleterious; [34]: likely pathogenic;	class 4, likely pathogenic

cDNA – coding DNA reference sequence, HGVS – Human Genome Variation Society, NFE – non-Finnish European, mRNA – messenger RNA, LOVD – Leiden Open Variation Database, IARC – International Agency for Research on Cancer, HDR – homology directed repair, mouse ES – embryonic stem cells

alternative splicing at the *BRCA1* might influence the significance of mutations in particular regions as well. According to several reports, the relative expression levels of the most abundant alter-

native transcripts $\Delta(9,10)$, $\Delta 11q$ and $\Delta(9,10,11q)$ are tissue-specific, cell-cycle regulated and markedly altered in tumour samples [53]. A few years ago a case was described of a woman iden-

tified with a bi-allelic mutation in *BRCA1* c.[594-2A>C; 2681_2682delAA] without symptoms of Fanconi anaemia, which led to the later revelation that c.594-2A>C was not a high-risk mu-

Tab. 11. *BRCA2* large intragenic rearrangements detected in Czech patients.

<i>BRCA2</i> NM_000059.3) exons affected	HGVS nomenclature	Number of families	Breakpoints; predicted change	LOVD-IARC class [4] // our class (if different or not specified)
deletion of exon 18	c.(7976+1_7977-1)_(8331+1_8332-1)del	1	breakpoints not characterized; detected by CNV analysis and confirmed by MLPA analysis; predicted r.(7977_8331del) => frame-shift	class 5, pathogenic
deletion of exons 19_24	c.(8331+1_8332-1)_(9256+1_9257-1)del	1	breakpoints not characterized; predicted at least: r.(8332_9256del) => frame-shift	class 5, pathogenic
complex rearrangement involving exons 21_27	c.[(8632+1_8633-1)_(8754+1_?)dup, (8886_9195)del, (?_9256+1)_(*1_?)dup]	1	duplication of Ex 21 and duplication from 3' end of Ex 24 to Ex 27 combined with 310bp deletion affecting the coding sequence from exon 22 to 5' end of Ex 24: c.8886_9195del (Fig. 1)	// class 4, likely pathogenic
duplication of exons 22_27	c.(8754+1_8755-1)_(*1_?)dup	1	breakpoints not characterized?	// class 4, likely pathogenic

HGVS – Human Genome Variation Society, LOVD – Leiden Open Variation Database, IARC – International Agency for Research on Cancer, CNV – copy number variations, MLPA – Multiplex Ligation-dependent Probe Amplification

tation [54]. The spliceogenic effect of c.594-2A>C was confirmed to upregulate viable Δ(9,10) in-frame isoform and a previously described pathogenic mutation specified as causing exon 10 skipping (a truncating alteration) was reclassified as a variant of VUS [54,55]. A naturally occurring isoform Δ(9,10) might be the cause of survival in a Fanconi anaemia-like case with bi-allelic *BRCA1* mutations c.594_597del (localised in exon 10) and p.Arg1699Trp [56]. Therefore we classified our novel frameshift mutation located in exon 10 of the *BRCA1* gene: c.622_625dup as likely pathogenic (class 4), because of the possibility to bypass this truncating mutation by a naturally occurring isoform Δ(9,10) and its putative hypomorphic effect.

The comprehensive description of *BRCA1* alternative splicing is highly relevant for diagnosis, in particular when assessing the impact of *BRCA1* germline variants on splicing. Recently in a consanguineous Danish family with several cases of breast/ovarian cancer a 58-year-old healthy homozygous carrier of the *BRCA1* c.4096+3A>G was identified which led to the reclassification of this splice site mutation to a 'variant of uncer-

tain significance' or even to a 'likely benign variant' [28]. *BRCA1* c.4096+3A>G has been shown to enhance the abundance of the naturally occurring isoforms: skipping of exon 11 and lacking 3309 nucleotides from exon 11 (Δ11q): c.[671_4096del,787_4096del]p.[Ala224_Leu1365del,Ser264_Leu1365del], which was previously assumed to affect function [27]. Currently, there is evidence that in-frame (naturally occurring) alternative transcripts may rescue gene functionality. If the cells are viable to overcome the loss of large segments of the coding sequences in the central part of the *BRCA1* gene in naturally occurring isoforms Δ(9,10), Δ11q and Δ(9,10,11q), it is unlikely that there could be any clinically significant missense mutation in this region. A potentially spliceogenic variant in the *BRCA1* gene c.4675+1G>A was confirmed to have residual full-length transcript [8] (Tab. 2). With the exception of one novel mutation all *BRCA* splice site mutations were characterised at the mRNA level (Tab. 2, 9). Novel *BRCA1* c.4185+1G>C was classified as likely pathogenic (class 4) on the basis of the prediction to cause exon skipping and confirmed exon skipping for several other variants detected in this

splice site: c.4185+1G>T, c.4185+1G>A, c.4185+2T>C, c.4185G>A (Tab. 2).

The use of MLPA to detect large-scale rearrangements is now a standard component of *BRCA1* and *BRCA2* gene testing in the clinical setting even if it is currently also widely used CNV analysis with next generation sequencing (NGS) data available. Genomic rearrangements accounted for 10.4% of all *BRCA* mutations detected in our study – 102 in *BRCA1*, 16 unique (Tab. 4), and only 4 in *BRCA2*, 4 unique (Tab. 11). Several breakpoints in *BRCA1* rearrangements were characterised [8] and a range of several deletions was confirmed by long-range PCR (Tab. 4). Genomic rearrangements are probably frequent in the *BRCA1* gene because of the extremely high density of intronic Alu repeats and the presence of a duplicated promoter region containing a *BRCA1* pseudogene that could provide hotspots for unequal homologous recombination [9]. However, genomic rearrangements in the *BRCA2* gene are very rare in our region. *BRCA2* deletion of exons 22 to 24 was detected by MLPA. The deletion of *BRCA2* exon 18 was originally detected by CNV analysis from NGS data and confirmed on two DNA samples by MLPA analysis. Suspected du-

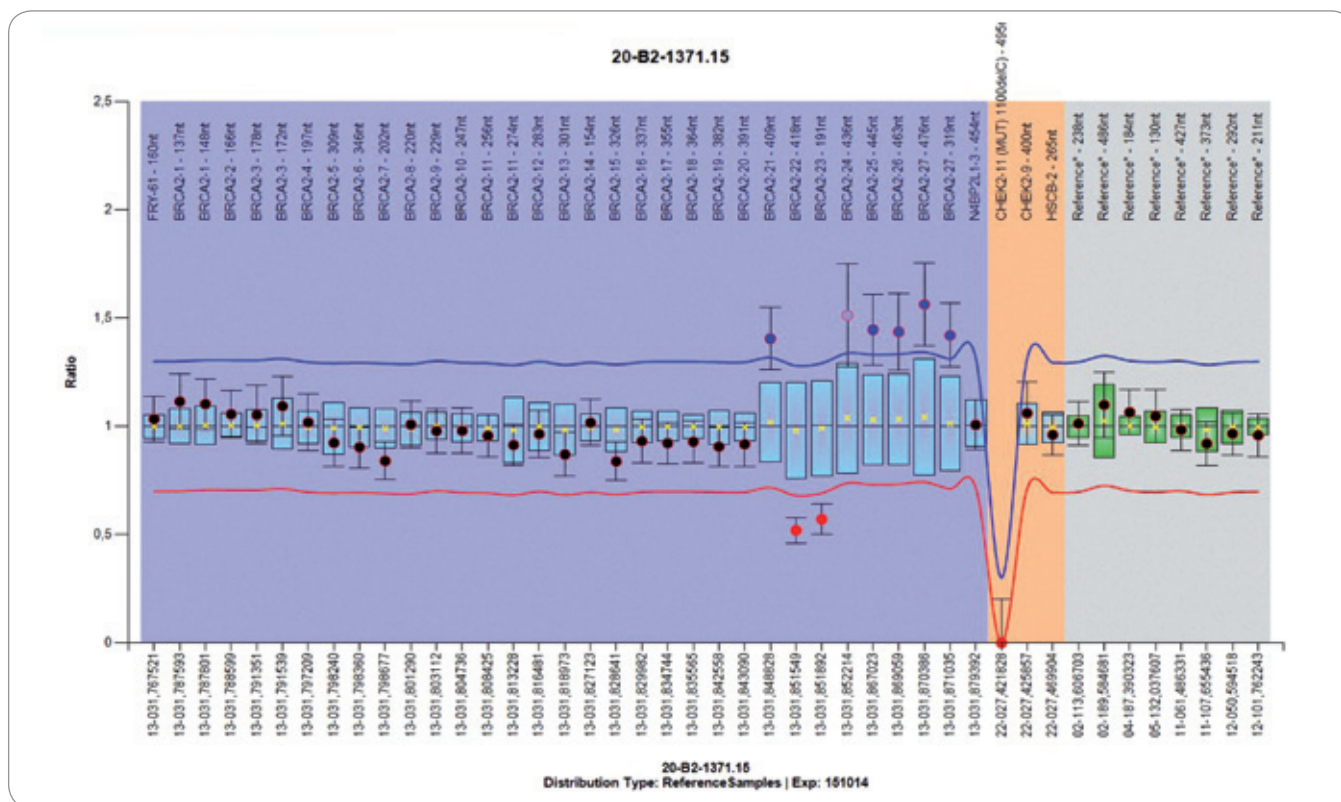


Fig. 1. Results of Multiplex Ligation-dependent Probe Amplification analysis using the Coffalyser software – family-96 with complex *BRCA2* rearrangements (identical profile detected in mother and in her son). Duplication of exon 21 and 3’ end of exon 24 to exon 27 combined with deletion affecting the coding sequence of exons 22 and 5’ end of exon 24: c.8886_9195del310 – confirmed by polymerase chain reaction analysis (amplified with primers for exon 21.Forward and exon 24.Reverse) and Sanger sequencing. *BRCA2* Probemix P045 (MRC Holland): probe 08267-L23772 of exon 24 (9455-9454 reverse GAAACGACAAAT-CCTATTAGTCC) corresponds to the systematic position c.9227-9228.

plication of *BRCA2* exons 22–27 come originally from the Children’s Clinic after comparative genomic hybridisation analysis beyond more 3’ distant region and was confirmed by MLPA. In one of the complex *BRCA2* rearrangements we have not been able to reveal the exact character of the changes (Fig. 1). MLPA analysis revealed a duplication of exon 21; deletion of exons 22 and 23 followed by duplication of exons 24 to exon 27. The deleted sequence was confirmed by PCR amplification and Sanger sequencing as c.8886_9195del310. The same MLPA profile (and CNV profile) was detected in two individuals – in a mother and in her son. Probably all detected *BRCA2* rearrangements are novel, not reported in public databases from other populations.

We may discuss whether MLPA will remain the standard technology to detect CNVs. Has it ever evaluated whether the

data generated by the NGS panel are robust enough to evaluate CNVs?

During the 20 years of *BRCA* analysis a number of variants, which were originally reported as of VUS, was gradually reclassified as likely benign or benign (supplementary Tab. 6, 7, 12, 13). As soon as information confirming the neutrality of any missense or intronic variant was available, we ceased to register their frequency and stopped reporting them. Reports indicate that common polymorphisms have not been included in the report and clinicians may not be aware of what common polymorphisms are in our population. Our clinicians were always informed when a variant was reclassified as pathogenic, but they were not always aware of the fact of neutrality. The laboratory’s interpretation has always been dependent on the information available at the time of the conclusion of the report. Thus, we decided to publish all detected

variants, even those already confirmed as neutral. This can serve clinicians in our region to review data in the patient records.

Some variants of VUS could not be classified unequivocally recently. Several new potentially significant missense variants have been detected for further analysis. The limited number of individual variants and lack of experimental validation lead to inconclusive interpretations but data sharing can help to speed up clarification of significance for some of them. Functional studies of potentially significant variants and population-level data with accurate phenotyping will improve variant classification and reduce uncertainties in future. By understanding more about the VUS interpretation, clinicians can help navigate medical decision-making using the best available information and become comfortable with accepting the fact that many DNA results cannot be interpreted with the tools and data available today.

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