**Charles University in Prague**

**First Faculty of Medicine**

Institute of Biochemistry and Experimental Oncology

**PhD Thesis – Short Report**

# GENETIC FACTORS RESPONSIBLE FOR BREAST AND OVARIAN CANCER DEVELOPMENT

## LARGE REARRANGEMENTS IN *BRCA1* AND *BRCA2* GENES

**Ivana Tichá**

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**PhD Programme:** Biochemistry and pathobiochemistry

**Program committee chairman:** Prof. MUDr. Jiří Kraml, DrSc.

**Supervising department:** Institute of Biochemistry and Experimental Oncology,
First Faculty of Medicine, Charles University in Prague
U Nemocnice 5, 128 53 Prague 2

**Author:** Mgr. Ivana Tichá

**Supervisor:** Doc. MUDr. Petr Pohlreich, CSc.

**Opponents:** . . . . . .

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## 2. Summary

**Background:** A greatly increased risk for development of hereditary breast cancer is associated with germline mutations in several susceptibility genes. In this study we analyzed large genomic rearrangements (LGRs) in *BRCA1/2* genes and we also focused on the role of *CHEK2* and *TP53* in tumorigenesis.

**Methods:** A series of 586 high risk patients with breast/ovarian cancer that had previously been tested negatively for small mutations in *BRCA1/2* was screened for LGRs by MLPA, LR-PCR and sequencing. Chromosome 17-specific aCGH was used to locate deletion breakpoints in regions flanking the *BRCA1* gene. MLPA-analysis was also used to detect two frequently occurring mutations in *CHEK2* (c.1100delC and a deletion of 5395 bp). The coding region of the *TP53* gene was analyzed by sequencing.

**Results:** We identified 9 different LGRs in the *BRCA1* gene in 16 patients. Five alterations (deletion of exons 1-17, 5-10, 13-19, 18-22 and 21-24) were novel. Deletions of exons 1-17, 5-14 and 21-22 were identified repeatedly, and represented population specific (founder) mutations. LGRs accounted for 12.1% (16/132) of all detected pathogenic *BRCA1* mutations. No LGRs were found in the *BRCA2* gene. Pathogenic mutations in other tested genes were less frequent; 2 were detected in *TP53* and 9 in *CHEK2*.

**Conclusions:** In our population, LGRs represent substantial proportion of pathogenic mutations in *BRCA1*. Our results indicate that screening for LGRs in *BRCA1* should include patiens from high-risk families as well as patients with non-familial cancer; in particular cases with early-onset breast cancer. Testing of *CHEK2* for the two recurrent mutations seems to be also relevant in our population; analysis of *TP53* may be restricted to cases of early onset breast cancer.

**Souhrn**

**Úvod:** Vývoj dědičného karcinomu prsu/ovaria podmiňují zárodečné mutace řady genů. V této studii byly analyzovány rozsáhlé přestavby genů *BRCA1/2* a dále jsme se zaměřili na úlohu genů *CHEK2* a *TP53* v tumorogenezi.

M**etody:** U 586 vysoce rizikových pacientů s karcinomem prsu/ovaria, u kterých nebyly standardní analýzou prokázány mutace v genech *BRCA1/2*, byly přestavby analyzovány pomocí MLPA, long range PCR a sekvenování; dlouhé delece přesahující hranice genu byly lokalizovány pomocí oligonukleotidové aCGH specifické pro lidský chromosom 17. MLPA analýza byla rovněž použita k detekci dvou často se vyskytujících mutací genu *CHEK2* (c.1100delC a delece 5395 bp). K analýze kódující oblasti genu *TP53* bylo použito sekvenování.

**Výsledky:** Detekovali jsme 9 různých přestaveb genu *BRCA1* u 16 pacientů. Pět z nich (delece exonů 1-17, 5-10, 13-19, 18-22 a 21-24) nebylo dosud popsáno. Delece exonů 1-17, 5-14 a 21-22 byly detekovány opakovaně a představují populačně specifické (founder) mutace. Dlouhé delece a přestavby představovaly 12,1% (16/132) všech detekovaných patogenních mutací genu *BRCA1*. V genu *BRCA2* žádná rozsáhlá přestavba nalezena nebyla. Výskyt patogenních mutací v dalších testovaných genech byl nižší; 2 mutace byly detekovány v genu *TP53* a 9 v genu *CHEK2*.

**Závěr:** Přestavby představují v naší populaci významnou část mutací genu *BRCA1*. Jejich analýza je dle našich výsledků indikována nejen u pacientek s rodinnou zátěží, ale i u sporadických případů onemocnění, především u pacientek s časným karcinomem prsu. Jako významné se rovněž jeví testování mutací genu *CHEK2*, které se specificky vyskytují v naší populaci; analýza genu *TP53* může být omezena na případy karcinomu prsu ve velmi nízkém věku (do 30 let).

# 3. Introduction

## 3.1 Pathogenesis of breast cancer

Breast cancer (BC) could be distinguished into two pathogenetically different forms: hereditary form – characterized by family history of BC – and sporadic form – without family history of cancer. Tumorigenesis of these two forms is different and employs various molecular mechanisms [1,2,3]. All genes involved in BC development are not known. Nevertheless, genes with defined role in BC development belong to tumor supressors, oncogenes or genes that play an important role in maintenance of genome integrity.

Knudson's two-hit theory explained the difference of tumorogenesis between the hereditary and sporadic forms of cancer [4]. In the hereditary form, one mutation (germline mutation) is inherited via the germ cells and the second (somatic mutation) occurs in somatic cells. In the nonhereditary form, both mutations occur in somatic cells. Individuals with germline mutation are predisposed to cancer development. In sporadic tumors both alleles of tumor suppressor gene have to be inactivated in the target cell, which is an unlikely event. Therefore this form of cancer is associated with later age of onset [4]. Study by Loman *et al.* [5] gives the evidence that BC occurred in younger age groups in *BRCA1* mutation carriers (typically before age 40) when compared to noncarriers.

Sporadic form is much more frequent and represents more than 90% of all BC cases while hereditary form include only 5-10%. Many genes important in BC pathogenesis were identified. Alterations in *BRCA1* and *BRCA2* genes are responsible for 60-80% of all hereditary cases and confer to the carrier a significatly higher risk of BC [6]. Germline mutations in genes *TP53* and *PTEN* are associated with rare cancer syndromes - Li-Fraumeni syndrome (*TP53*) and Cowden syndrome (*PTEN*) – characterized by a significantly increase risk of BC development.

The heritable effects of genes in human cancer pathogenesis range from high penetrance with an attendant high likelihood of developing cancer to low-penetrant genes with an attendant increased risk of causing cancer, albeit less likely than that for high-penetrant genes. BC and ovarian cancer (OC) risk in *BRCA1* and *BRCA2* mutation carrier is high (high-penetrant genes) whereas mutations in other genes such as *ATM* (low-penetrant gene) and *CHEK2* (middle-penetrant gene) only slightly increase disease risk [6,7,8,9].

### 3.2 Large rearrangements in *BRCA1* and *BRCA2*

*BRCA1* (OMIM 113705) and *BRCA2* (OMIM 600185) are major predisposition genes in hereditary breast and ovarian cancer. Frequency of germline mutations in these genes was estimated at 2-4.7% in unselected BC patients [10,11,12]. Pathogenic alterations are represented predominantly by small deletions, insertions and point mutations leading to premature termination of translation and production of truncated proteins. Numbers of large genomic rearrangements (LGRs) of these genes have been increasingly reported by several groups [13,14] during last decade following the introduction of multiple ligation-dependent probe amplification (MLPA) analysis [15]. LGRs include deletions or duplications of large genomic regions or alterations that combine both deletion and insertion events. LGRs are much more common in *BRCA1* than *BRCA2*, which is attributed to higher density of *Alu* repetitive sequences in *BRCA1* locus (42%) compared to *BRCA2* locus (20%) [16,17]. *Alu* repeats could be implicated in unequal homologous recombination event leading to deletion or insertion of large DNA sequence. Most of *BRCA1* LGRs are the result of unequal homologous recombination between *Alu* repeats [13,14] while non-homologous events are more comon in *BRCA2* LGRs [13]. The recombination events that involve homologous regions located in the *BRCA1* gene and the *BRCA1* pseudogene (*ψBRCA1*) were described and later characterized by Puget and colleagues [18,19] and subsequently also by other authors [13,14]. The identification of numerous recombination sites in the homologous regions of *BRCA1* and *ψBRCA1* indicates that these regions represent hot spots for recombination [18].

The majority of LGRs at the *BRCA1* locus are unique (22/29 reported by the year 2005; 53/81 reported by the year 2010) [13,14]. They have been studied in several countries and predominantly have been reported to account for about 8-10% of all detected *BRCA1* pathogenic mutations [14,19,20,21,22,23]. The elevated incidence of LGRs in certain ethnic groups is caused by a founder effect. The most remarkable example has been found in the Dutch population, in which deletions of exon 13 or 22 represented 36% (12/33) of all *BRCA1* mutations found in 170 families [24]. In another group of 805 Dutch patients, these two deletions were detected in 23% (28/121) of the *BRCA1* positive cases and all LGRs accounted for 27.3% of *BRCA1* mutations [15]. Another founder mutation is the exon 13 duplication which was first described in one Portuguese and three American families [25] but subsequently has been identified in several populations [26]. High proportion of LGRs was also reported from Italy, where LGRs accounted for 19% of the *BRCA1* mutation positive families [27].

Contrary to *BRCA1*, the germline *BRCA2* rearrangements have not been identified in several populations [28,29,30] and in other geographic areas contributed to a small proportion of hereditary BC and OC cases [21,23,31,32]. LGRs in the *BRCA2* gene were most frequent in high-risk families with multiple BC cases including one or more male BC cases [33,34].

### 3.3 Detection of LGRs

Several methods were developed for the detection of deletions or duplications that may span tens of thousands bases or whole genes.

First rearrangements were identified using Southern blot analysis, combined with RNA analysis methods such as RT-PCR and protein truncation test (PTT). [19,24,35,36, 37,38,39,40,41]. However, Southern blot analysis is a time consuming method and a lot of genetic material is needed. Analyses that examine cDNA could be influenced by aberrant mRNA degradation also known as nonsense mediated decay [42]. Nevertheless, several LGRs were detected during cDNA analysis [43,44].

The later high-throughput DNA-based techniques were used for detecting copy number changes in regions of interest. To these methods belong semiquantitative multiplex PCR [45], multiplex ligation-dependent probe amplification (MLPA) [46] and fluorescence DNA microarray assays [47].

The most common technique for screening genomic deletions and duplications in a wide range of genes is MLPA (www-mrc-holland.com) [46] which is widely used in diagnostic practice. MLPA is a multiplex PCR method detecting abnormal copy numbers of amplified fragments. The method is based on hybridization of a pair of adjacent oligonucleotide probes to the target sequence. Ligation of probes is a necessary step for amplification. Another hybridization method used in this regard is array-based comparative genomic hybridization (aCGH). Oligonucleotide aCGH is relatively new technique that also enables the search for copy number alterations. Contrary to MLPA, aCGH gives information about borders of deleted or duplicated region.

## 4. Hypothesis and aims of study

Analysis of genes susceptible to hereditary breast cancer has been performed at our laboratory since 1998. Spectrum of mutations in major predisposition genes *BRCA1/2* was characterized and contribution of *BRCA1/2* mutations to BC development in our population was revealed. Population specific mutations of these genes were identified and mutation analysis was optimized [48,49]. However, causal mutations in *BRCA1/*2 were detected only in a part of high-risk families.

LGRs of *BRCA1* gene (in high-risk patiens) were described for the first time in Czech Republic in 2007 [50]. In this study LGRs represented 10.4% (10/96) of all identified *BRCA1* mutations [50]. One *BRCA1* rearrangement was also identified by RNA based analysis performed in our laboratory, which also implied significant role of LGRs in hereditary breast cancer [44].

The main aim of this study was to analyze large rearrangements in *BRCA1* and *BRCA2* loci in high-risk breast and ovarian cancer patients from the Prague area.

MLPA analysis was applied for screening of more than 580 patients negatively tested for *BRCA1/2* point mutations. The intention of the study was to assess frequency and distribution of LGRs in a relatively large group of patients and to characterize the breakpoints in all gene alterations Finally, reliable inclusion criteria for testing of LGRs in *BRCA1/2* in Czech population should be assessed.

The group of patients was further subjected to mutation analysis of *TP53* and *CHEK2* genes to assess their impact in BC development in Czech high-risk BC and OC patients.

## 5. Material and methods

### 5.1 Patients

Selection for genetic testing was performed between 2002 and 2009 at the Institute of Biology and Medical Genetics, at the Department of Oncology and at the Department of Obstetrics and Gynecology, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague. The protocol of investigation was approved by the Ethical Committee of the first Faculty of Medicine and the General University Hospital and all participants gave their written informed consent prior to genetic testing.

All patients were of Czech ancestry and major part of them comes from Prague region. A series of 740 patients was first analyzed for the presence of point mutations and small deletions or insertions in *BRCA1/2* genes as described previously [48,49] and those tested negative for mutations in coding gene regions (586 cases) were subsequently screened for LGRs at the *BRCA1/2* loci and also for mutations in two other predisposition genes, *TP53* and *CHEK2*. The group of 586 patiens included 276 patients from high-risk cancer families (hereditary/familiar cases) and 310 high-risk patients with sporadic tumors (non-familiar cases).

The group of high-risk cancer families was selected according following criteria in first- or second-degree relatives: (1) two cases of either BC diagnosed before the age of 50 or OC diagnosed at any age; (2) three or more cases of breast or ovarian cancer diagnosed at any age. A total of 186 families had a history positive for BC only (HBC families), 80 families had both BC and OC (HBOC families) and 10 families had OC only (HOC families). The second group of high-risk sporadic cases included patients diagnosed with BC before the age of 36 (147 cases) or OC before the age of 40 (69 cases), patients with bilateral BC before the age of 51 (29 cases), patients with both primary breast and ovarian cancer (19 cases) or medullary breast carcinoma (20 cases) and cases of male BC (17 cases). This group includes also 9 cases with triple negativity of receptors (ER, PR and HER2/neu).

### 5.2 General methods

DNA and RNA isolation

Primer design

Polymerase chain reaction (PCR)

RT-PCR (Reverse transcription PCR)

DNA electrophoresis in agarose gel

Automatic sequencing

### 5.3 MLPA analysis

The MLPA analysis was verified using SALSA MLPA kit P103 for testing *DPYD* gene (dihydropyrimidine dehydrogenase; OMIM 612779) for the presence of LGRs [51]. Mutations in *DPYD* coding region have been associated with severe toxicity in patients treated with fluoropyrimidines (FP). Point mutations represents the majority of gene alterations but fragile site within the *DPYD* locus, which could be prone to breaks and rearrangements, was mapped recently [52]. Collected DNA samples of cancer patiens with FP-toxicity were used for analysis.

The SALSA MLPA kits for *BRCA1* (the primary screening kit P002B and the confirmatory kit P087) and *BRCA2* (kit P045-B1) (MRC-Holland, Amsterdam, The Netherlands) were used for quantification of each of the *BRCA1*/*2* exons. The assay was done according to the manufacturer's instructions except that half volume reactions and 100 ng of genomic DNA were used. Amplified products were separated in a 36 cm capillary filled with POP-7 polymer on an ABI 3130 genetic analyzer (Applied Biosystems, Foster City, USA). The collected data were analyzed using Gene Mapper *v*.4.0 Software (Applied Biosystems) and evaluated by both visual examination of the peak profiles and quantitative comparison of peak areas using Coffalyser program reccomended by the manufacturer (http://www.mlpa.com/coffalyser). Samples differing in a relative peak area by more than 30% were reanalyzed. Samples with single exon deletions were sequenced to exclude the presence of sequence variants affecting probe hybridization.

### 5.4 Array-based comparative genomic hybridization

Chromosome 17-specific aCGH (Roche NimbleGen, Madison, USA) was used for measuring copy number differences between test and control DNA samples at the *BRCA1* locus. Analysis was performed to locate deletion breakpoints in regions flanking the gene. The arrays format was 385K; median oligonucleotide probe (50 to 75mer) spacing was 160 bp.

Sample DNA and control DNA (from blood samples of 100 random blood donors) were sonicated to produce fragments of approximately 500 to 2,000 bp and further processed according to the supplier's protocol (Roche NimbleGen). Briefly, 1 µg of sample DNA was labeled with Cy3- and the same amount of control DNA with Cy5-random nanomer using a NimbleGen Dual-Color DNA Labeling Kit. Differentially labeled DNA samples were purified, combined (6 µg of each DNA) and hybridized to arrays for 18 hours at 42°C. The slides were washed, dried and fluorescence was recorded at 3µm resolution using microarray

fluorescent scanner Innoscan 700 (INNOPSYS, France). Scanned images were analyzed using NimbleScan software. Fluorescence signals were normalized with qspline algorithm [53] to compensate for inherent differences in signal between the two dyes. Copy number analysis was performed using the segMNT algorithm [54], available in NimbleScan software, and statistically significant regions of aberration were visualized using SignalMap software (Roche NimbleGen).

### 5.5 DNA breakpoint analysis

Large deletions detected by MLPA and located by aCGH were validated by identification and characterization of the genomic breakpoints. Long-range PCR (LR-PCR) was performed according to the manufacturer's instructions (Expand Long Template PCR System, Roche Applied Science) with primer pairs flanking the breakpoints to amplify the junction fragment carrying the deletion. Amplified PCR fragments were separated on agarose gels and visualized by ethidium bromide staining. A shorter PCR product, corresponding to the deleted allele, was obtained from DNA samples of all mutation carriers identified by MLPA analysis. Such aberrant PCR products were purified with ExoSAP-IT (USB Corp., Cleveland, USA) according to the manufacturer's instructions and sequenced with appropriate primers using the BigDye *v*.3.1 terminator cycle sequencing kit (Applied Biosystems) on an ABI Prism 3130 genetic analyzer (Applied Biosystems). Data were depicted with the Sequencing Analysis software (Applied Biosystems).

### 5.6 Haplotype analysis

DNA samples carrying deletion of exons 1-17, 5-14 or 21-22 in the *BRCA1* gene were genotyped for two *BRCA1* intragenic microsatellite markers (D17S855, D17S1322 or D17S1323) and two markers (D17S1320, D17S1325) flanking *BRCA1* gene. Each marker was amplified by PCR using FAM-labeled forward primers and analyzed on an ABI Prism 3130 genetic analyzer.

## 6. Results and discussion

### 6.1 Prevalence of genomic rearrangements in BRCA1/2 genes

LGRs were examined in 586 unrelated high-risk patients negative for *BRCA1* and *BRCA2* point mutations [55]. MLPA analysis revealed 9 different LGRs in *BRCA1* gene in 16 (2.7%, 16/586) probands. Specification of families with identified mutations and characterization of LGRs are described in Table 1. The frequency of LGRs in the whole group of 740 *BRCA1/2* tested patients (including 154 patients with point mutations) was 2.2% (16/740). Deletions were detected in the entire *BRCA1* locus and varied in size from less than 4 kb to more than 80 kb. Five different LGRs were novel. Only two of the identified mutations (deletion of exons 1-2 and duplication of exon 13) occurred repeatedly in several countries [13,14,26] and two other mutations (del ex 5-14 and del ex 21-22) have already been detected in the Czech population [50,44]. On the contrary, no LGRs were identified in the *BRCA2* gene. Very low frequency of LGRs was reported from Germany and Denmark where LGRs in the *BRCA2* gene were found only in one family with male BC out of 450 and 642 analyzed families, respectively [21,23]. Higher frequency of rearrangements in the *BRCA2* gene was found in Spain (2/20) [33] and France (3/39) [34] in families with female and male BC. In our study, only a small group of 5 families with female and male BC was analyzed with negative results for rearrangements in *BRCA2*. On the other hand, our results are not in agreement with analyses performed in France [56], Spain [33] and America [43] where genomic rearrangements of the *BRCA2* gene have been found in families with no cases of male BC.

*BRCA1* LGRs were more frequent in the group of hereditary cases (14/276, 5.1%) than in the group of sporadic cancer cases (2/310; 0,6%; P = 0,0008) and the frequency was higher among patients from HBOC + HOC families (7,8%; 7/90) than among patients from HBC families (3,8%; 7/186; P = 0,16). Similarly, the highest frequency of point mutations was found in a group of HBOC + HOC families (Table 2). Among sporadic cancer cases, two LGRs at the *BRCA1* locus were found in a woman diagnosed with medullary breast carcinoma at the age of 39 and in a woman with OC at the age of 36.

In total, 16 *BRCA1* LGRs accounted for 12.1% (16/132) of all identified *BRCA1* mutations in a group of 740 analyzed families (including 138 families with small mutations in *BRCA1/2*) (Table 2). The frequency of LGRs was lower in our study than that observed in the Netherlands (27.3%) [15] and Italy (19%) [27], but it was in agreement with data reported from Denmark (12.5%) [23], France (9.5%) [22], Germany (9.6%) [21] and Spain (8.2%) [57]. Frequency of LGRs at the *BRCA1* locus (10.4%; 10 of 96 detected *BRCA1* mutations in

290 patients) described in the east part of the Czech Republic (the Masaryk Memorial Cancer Institute, Brno) [50] was similar to our results. However, only two mutations (deletion of exons 5-14 and 21-22) were identical in both parts of the country. Deletions of exons 1-17 and 5-14 in the *BRCA1* gene are probably Czech-specific mutations and comprised 50% (8/16) of all identified LGRs. While the occurrence of exon 1-17 deletion was restricted to the Prague area so far, the deletion of exons 5-14 was also detected four times in Brno and represented the most frequent LGR in the Czech Republic. The complex rearrangement involving exon 21-22 deletion was identified two times in Brno and also in our laboratory and may represent further population specific mutation. To evaluate whether repeatedly occurring deletions of exons 1-17, 5-14 and 21-22 represent founder mutations, haplotype analysis was performed in families carrying these deletions. One specific ~857 kb haplotype defined by D17S855, D17S1322, D17S1323, D17S1320 and D17S1325 markers was common for each carrier with a given type of alteration, which indicates the same origin of each mutation. On the contrary, the same combination of alleles was not found in 20 control samples.

The deletion of exons 1-2 and duplication of exon 13 were the only identified mutations spread throughout Europe and America [13,14]. Frequent occurrence of unique mutations is in agreement with the results obtained from other countries [13,14].

### 6.2 Mechanisms of LGRs

Homologous recombinations between *Alu* sequences are associated with most reported rearrangements at the *BRCA1* locus [13,14]. High percentage of LGRs identified in our study was also caused by recombinations of *Alu* repeats (Table 1). Deletions of exons 5-14, 13-19 and 21-24 were not mediated by *Alu*/*Alu* recombination and the deletion of exons 1-2 resulted from recombination of homologous sequences in intron 2 of *BRCA1* and intron 2 of *ΨBRCA1* gene. Such recombination generates the chimeric gene consisting of *BRCA1* exons 3-24 fused to exons 1-2 of its pseudogene that lacks the *BRCA1* translation initiation codon and the *BRCA1* promoter [18]. The affected allele is probably not transcribed because of a promoter deletion [18,58].

***Table 1:*** Germline LGRs in the *BRCA1* gene.

| Exon | chromosomal breakpoints[a], mutation designation - gDNA[b/c] mutation designation - cDNA[d] | Predicted mutation effect | Recombination | Patient No | BC & OC cases in family[e] (age at diagnosis) | Other tumors | Ref. |
|---|---|---|---|---|---|---|---|
| **1-2** | [a] chr17: 38,525,493-38,562,426<br>[d] c.1-32787_80+4067del36934<br>[b] g.197575_234508del36934 | No tanscript? | *ΨBRCA1*/*BRCA1* | 779 | BC (**58***); OC (**51***, 47) | - | [13, 18, 21] |
| **1-17** | [a] chr17:38,470,578-38,551,073<br>[d] c.1-21434_5075-1084del80496<br>[b] g.208928_289423del 80496 | Loss of ~90% coding sequence | *Alu*Y/*Alu*Y | 206<br>225<br>269<br>416 | BC (**28**, 50, 50**)**<br>BC (27); OC (**40**)<br>BC (**35**, 36)<br>BC (39. 62*); OC (**42**, 64*) | Uterus (3x)<br>Colon, lung<br>-<br>Lymphoma | novel |
| **5-10** | [a] chr17:38,501,028-38,516,581<br>[d] c.135-4505_670+361del15552ins35<br>[c] g.17696_33247del 15552ins35 | p.Lys45Asn fsX4 | *Alu*Sx/*Alu*Y/*Alu*Jb | 1190 | BC (50, 50); OC (**46**) | - | novel |
| **5-14** | [a] chr17:38,480,980-38,512,561<br>[d] c.135-485_4485-913del31583<br>[c] g.21716_53298del31583 | p.Lys45_Lys1495del | LINE/- | 278<br>453<br>862<br>938 | BC (**40/41**, 63, 69, 35/35)<br>BC (**39**)<br>BC (**44**, 48*); OC (60, 64*)<br>BC (**32**, ?*, ?); OC (?*) | Pancreas,uterus<br>-<br>Prostate<br>- | [21, 50] |
| **13** | [a] chr17:38,483,825-38,489,905<br>[d] c.4186-1787_4358-1669dup6081<br>[c] g.44369_50449dup6081 | p.Val1454Glu fsX7 | *Alu*Sx/ *Alu*Sx | 1388 | BC (**59**), OC (57) | - | [25] |
| **13-19** | [a] chr17:38,467,226-38,492,424<br>[d] c.4185+4062_5193+1650del25201<br>[c] g.41850_67050del25201 | p.Gln1396_Glu1731del | -/*Alu*Sx | 571 | BC (**56/56**, 48, 44) | - | novel |
| **18-22** | [a] chr17:38,454,318-38,470,629<br>[d] c.5075-1135_5046+347del16316<br>[c] g.63647_79962del16316 | p.Asp1692Gly fsX27 | *Alu*Y/*Alu*Sx | 897 | BC (**35**, 64,?,?,?,?) | - | novel |

***Table 1:*** Germline LGRs in the *BRCA1* gene.

| Exon | chromosomal breakpoints[a], mutation designation - gDNA[b/c] mutation designation - cDNA[d] | Predicted mutation effect | Recombination | Patient No | BC & OC cases in family[e] (age at diagnosis) | Other tumors | Ref. |
|---|---|---|---|---|---|---|---|
| **21-22** | [a] chr17:38,453,374-38,457,152<br>[d] c.5278-492_5407-128del3779ins236<br>[c] g.77128_80906del3779ins236 | p.Ile1760_Thr1802del | *Alu*Sx/*Alu*Jb | 102<br>1293 | OC (**<u>36</u>**)<br>BC (**<u>41</u>**, ?,?) | -<br>- | [44, 50] |
| **21-24** | [a] chr17:38,433,150-38,458,787<br>[d] c.5278-2135_5592+18067del25639insTAG<br>[c] g.75489_101127del25639 insTAG | No tanscript?<br>p.Ile1760fs | -/- | 609 | BC (**<u>38</u>**, ?,?) | - | novel |

[a] Genomic locale for chromosome 17is from the UCSC genome browser, Mar 2006 assembly; [b] Reference sequence: GenBank NC_00017.9 complement (38300000..38760000); [c] Reference sequence: GenBank L78833.1; [d] Mutation numbering: +1 corresponds to the A of the ATG translation initiation codon in the reference sequence U14680.
BC – breast cancer, OC – ovarian cancer; Age of onset of disease in proband *in bold unerlined* numbers; asterisks (*) mark the patient with both BC and OC; ages at diagnosis of bilateral BC cases are separatek by a slash (/).

***Table 2:*** Frequency of genetic changes in *BRCA1/2* loci in relation to classification of patients and families.

| Classification | Number of cases | Mutations in *BRCA1* (%) | Mutations in *BRCA2* (%) | LGRs in patients in the study (%) | LGRs in patients negatively tested for *BRCA1/2* mutation (%) |
|---|---|---|---|---|---|
| Hereditary cases | 389 | 81 (20,8) | 32 (8,2) | 14 (3,6) | 14/276 (5,1) |
| HBC | 238 | 32 (13,4) | 20 (8,4) | 7 (2,9) | 7/186 (3,8) |
| HBOC+ HOC | 151 | 49 (32,5) | 12 (7,9) | 7 (4,6) | 7/90 (7,8) |
| Sporadic cases | 351 | 35 (10,0) | 6 (1,7) | 2 (0,6) | 2/310 (0,6) |
| Overall | 740 | 116 (15,7) | 38 (5,1) | 16 (2,2) | 16/586 (2,7) |

HBC – hereditary breast cancer syndrome, HBOC – hereditary breast and ovarian cancer syndrom; HOC – hereditary ovarian cancer syndrome

### 6.3 Using of chromosome 17-specific aCGH

Sequencing of the junction fragment amplified by LR-PCR that confirms MLPA results and characterizes deletion breakpoints is a necessary step in the LGR analysis. However, location of the breakpoints and PCR amplification may fail when the deletion interferes with large introns or regions flanking the gene. In tested carriers, we initially failed to amplify specific junction fragments in two novel LGRs with borders located in regions adjacent to the *BRCA1* gene (deletions comprising exons 1-17 and 21-24). Chromosome 17-specific aCGH with a high density of oligonucleotide probes is a method that enables accurate mapping of the deleted region (Figure 1). The technique was evaluated by analysis of a sample with previously identified breakpoints (del ex 18-22) (Figure 1b) and consequently two novel *BRCA1* rearrangements with unknown boundaries were analyzed (Figure 1a,c). In all cases, the range of the deletion was accurately mapped, which enabled convenient PCR design and amplification of a short junction fragments (less than 1,500 bp) suitable for sequence analysis (Figure 1). The sample No 609 with deleted exons 21-24 (del of 25,639 kb) was tested by both chromosome 17-specific (median probe spacing ~160 bp) and whole-genome aCGH (median probe spacing ~7000 bp) (Roche NimbleGen). The deletion was identified by chromosome 17-specific aCGH, while the resolution power of whole-genome aCGH with lower density of oligonucleotide probes was not satisfactory (data not shown).

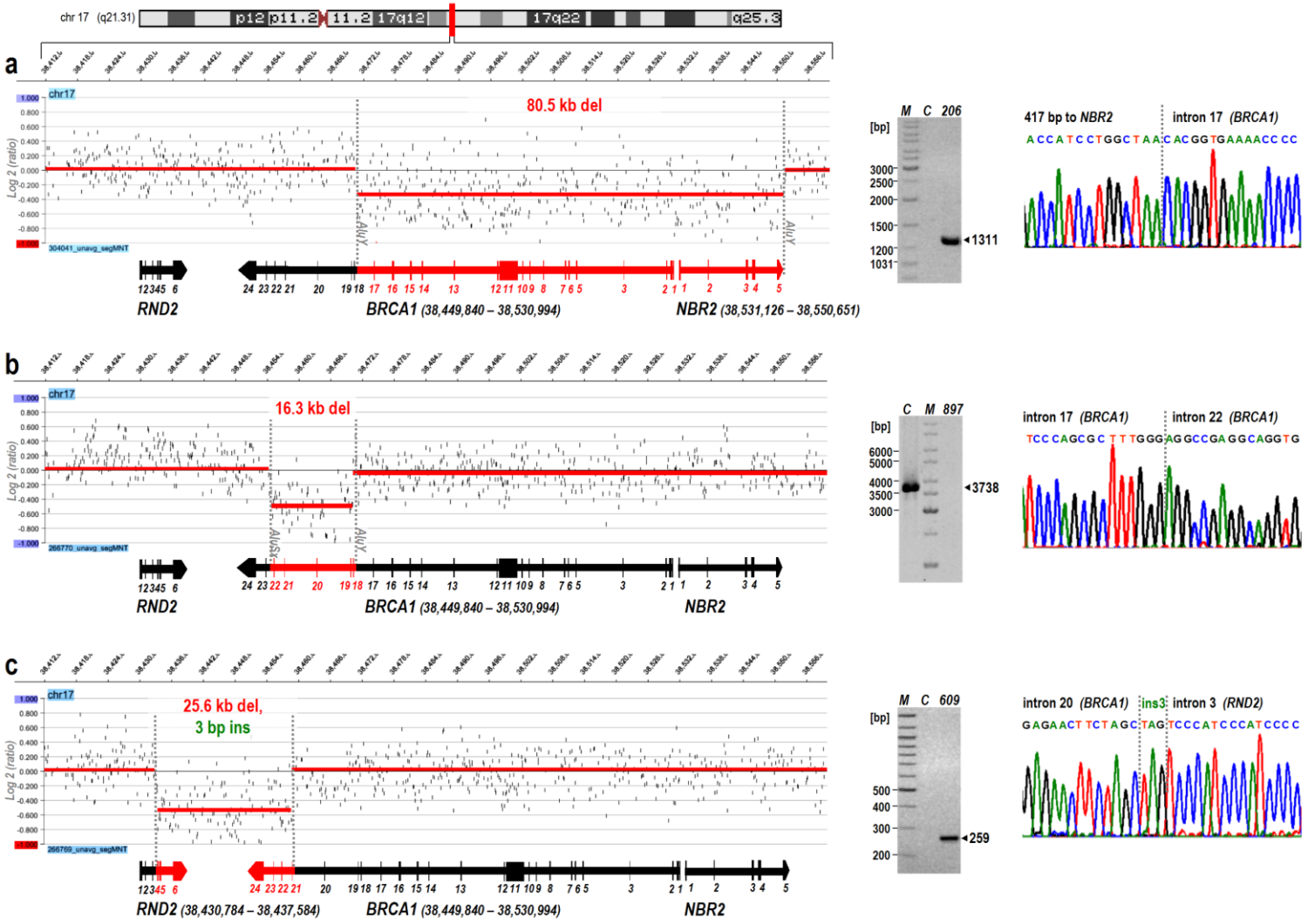


***Figure 1:*** Characterization of breakpoints of three novel genomic rearrangemets using chromosome 17-specific aCGH, LR-PCR and sequencing.
***Left*** - detection of copy number changes at the *BRCA1* locus by aCGH. *Red lines* represent the results of segMNT analysis. Deleted regions are shown by *lower red lines*. The schemes give the position and orientation (indicated by *arrows*) of *BRCA1*, *NBR2* and *RND2* genes and position of deleted regions of genes (*in red*). Genomic locale is from the UCSC genome browser, Mar 2006 assembly.
***Right*** - analysis of PCR products on agarose gels and sequence verification characterizing breakpoints. Breakpoints are indicated by dashed lines.
(a) Alu-mediated 80.5-kb deletion comprising exons 1-17 and the whole NBR2 gene;
(b) Alu-mediated 16.3-kb deletion comprising exons 18-22;
(c) a rearrangement combining a 25.6-kb deletion of exons 21-24 and the last three exons of RND2 gene and insertion of three nucleotides.

### 6.4 Mutation analysis of *TP53* and *CHEK2* genes

A group of 586 patients was further tested for mutations in the coding region of *TP53* and for two *CHEK2* mutations (c.1100delC and 5395 bp long deletion spanning exons 8-9). Mutations in *TP53* were identified by PCR and direct sequencing, in CHEK2 by MLPA and sequence analysis.

*TP53* belongs to high-penetrant genes responsible for hereditary breast and ovarian cancer.

***Tabulka 3:*** Pathogenic germline mutations in *TP53*.

| Exon | mutation designation gDNA[a] cDNA[b] | Predicted mutation effect | Patient No | BC & OC cases in family[c] (age at diagnosis) | Other tumors |
|---|---|---|---|---|---|
| **8** | [a] g.14487G>A [b] c.818G>A | p.Arg273His | 192 | BC (**28**, 30) | – |
| **8** | [a] g.14484 T>G [b] c.815T>G | p.Val272Gly | 1071 | BC (**27**, 49) | lung (52) |

[a] Reference sequence: GenBank X54156; [b] Mutation numbering: +1 corresponds to the A of the ATG translation initiation codon in the reference sequence NM_000546; [c] BC – breast cancer, OC – ovarian cancer; Age of onset of disease in proband *in bold unerlined* numbers; asterisks (*) mark the patient with both BC and OC.

Germline mutations of the *TP53* gene are often associated with Li-Fraumeni syndrome (OMIM 151623), which is a rare cancer syndrome characterized by multiple tumors within an individual, and multiple affected family members.

We found two pathogenic mutations of *TP53* in very young patients (before the age of 38 years) from families with no Li-Fraumeni features (Table 3). Mutations of this gene accounted for 0.3% (2/740) of all analyzed breast and ovarian cancer cases.

Mutation analysis of the *CHEK2* gene was restricted to the two recurrent clinicaly important mutations (c.1100delC and deletion of exons 8-9 [43,59]. Moreover deletion of exons 8-9 was found in the Czech Republic and likely belongs to Slavic founer mutations [43]. These mutations were found in 1.2 % (9/740) of all high risk patients (Table 4).

***Tabulka 4:*** Germline mutations in *CHEK2*.

| Exon | mutation designation<br>gDNA[a]<br>cDNA[b] | Predicted mutation effect | Patient No | BC & OC cases in family[c] (age at diagnosis) | Other tumors |
|---|---|---|---|---|---|
| **8-9** | [a]g.39870_45264del5395<br>[b]c.909-2028_1095+330del5395 | p.Met304LeufsX16 | 323 | BC (**56**, 40) | stomach, rectum, prostate |
| | | | 435 | BC (**33**, 60) | – |
| | | | 604 | BC (**51**, 50) | – |
| | | | 900 | BC (**43**) | nodes |
| | | | 1168 | BC (**53/53**) | Hodgkin lymphoma (at 14) |
| **10** | [a]g.45966delC<br>[b]c.1100delC | p.Thr367MetfsX15 | 284 | OC (**18**) | – |
| | | | 931 | BC (**32**, ?) | – |
| | | | 943 | BC (**35**) | – |
| | | | 1079 | BC (57, 38) | prostate |

[a] Reference sequence: GenBank NG_011520.12 complement (8474298..8528391); [b] Mutation numbering: +1 corresponds to the A of the ATG translation initiation codon in the reference sequence NM_007194.3; [c] BC – breast cancer, OC – ovarian cancer; Age of onset of disease in proband *in bold unerlined* numbers; male probands are *in red*; ages at diagnosis of bilateral BC cases are separatek by a slash (/).

## 7. Conslusions

The incidence of breast cancer is steadily increasing in the Czech Republic. Tumor development is a result of accumulation of gene alterations. Tumorigenesis is associated particularly with genes involved in regulation of cell cycle and genome stability. Hereditary carcinomas account for ~5-10% of all breast tumors and 60-80% of them are caused by a germline mutation in the major predisposition genes *BRCA1* and *BRCA2*. Other genes, such as *TP53* and CHEK2, play a role in breast cancer pathogenesis.

Analysis performed in 586 high-risk families and patients with sporadic cancer demonstrated that LGRs at the *BRCA1* locus account for 12.1% of all disease causing mutations in the *BRCA1* gene. The two Czech-specific founder mutations, the deletion of exons 1-17 and 5-14, were dominant gene alterations. The deletion of exons 21-22 is probably another Czech founder mutation. Our results indicate that in the Czech population, the screening for LGRs in the *BRCA1* gene in high-risk BC and/or OC families should be supplemented with screening of patients with non-familial cancer, particularly in cases with early-onset breast or ovarian cancer and cases of medullary BC. On the contrary, our analyses do not support the need to screen for LGRs in the *BRCA2* gene. Screening may currently be restricted to high-risk families with male breast cancer, which were found positive for large deletions in *BRCA2* in many other studies. Characterization of deletion

breakpoints belongs to essential steps in analysis of LGRs. Chromosome-specific aCGH is a method suitable for accurate detection of deletion boundaries, which markedly facilitates the design of specific primers for amplification of a junction fragment and sequence analysis.

Mutation analysis of coding region of *TP53* and MLPA screening for the two recurrent *CHEK2* mutations (c.1100delC and deletion of 5395 bp comprising exons 8-9) revealed that especially analysis of *CHEK2* gene could be clinically relevant. Pathogenic *TP53* mutations were found in very young women from HBC families without features of Li-Fraumeni syndrome.

To sum up, LGRs represent considerable part of *BRCA1* mutations and criteria for screening were declared. Genes *TP53* and *CHEK2* have a role in breast cancer development in a minority of high-risk patients in our population.

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## 9. List of author´s publications, presentations and award

### 9.1 Publications in extenso related to PhD Thesis

**Tichá I**, Kleibl Z, Stříbrná J, Kotlas J, Zimovjanová M, Matějů M, Zikán M, Pohlreich P (2010) *Screening for genomic rearrangements in BRCA1 and BRCA2 genes in Czech high-risk breast/ovarian cancer patients: high proportion of population specific alterations in BRCA1 gene.* Breast Cancer Res Treat. Epub 2010 Feb 5 [Epub ahead of print]

(**$IF_{2008}$ = 5.684**)

**Tichá I**, Kleiblová P, Fidlerová J, Novotný J, Pohlreich P, Kleibl Z: *Lack of large intragenic rearrangements in dihydropyrimidine dehydrogenase (DPYD) gene in fluoropyrimidine-treated patients with high-grade toxicity.* Cancer Chemother Pharmacol. 2009 Aug;64(3):615-8. Epub 2009 Mar 14

(**$IF_{2008}$ = 2.740**)

#### 9.1.1 Published abstracts

**Ticha I**, Soukupová J, Janatova M, Kleibl Z, Pohlreich P (2008) *The role of alterations in BRCA1, BRCA2, TP53 and ATM genes in sporadic breast tumors*. EJC Suppl. 6:118.

Pohlreich P, Kleibl Z, Stříbrná J, Soukupová J, **Tichá I**, Matějů M, Zikán M, Novotný J, Kotlas J, Sevcik J (2008) *Screening for inherited mutations in the Czech high risk breast cancer patients – analysis of 400 families*. EJC Suppl. 6:118.

**Tichá I**, Stříbrná J, Soukupová J, Janatová M, Kleibl Z, Havranek O, Pohlreich P (2010) *Genomic rearrangements in BRCA1/2 and CHEK2 genes in Czech high-risk breast/ovarian cancer patients.* EJC Suppl. 8:22.

Pohlreich P, Stříbrná J, **Tichá I**, Soukupová J, Kliebl Z, Zikán M (2010) *Predisposing genes in hereditary breast and ovarian cancer in the Czech Republic.* EJC Suppl. 8:16.

Soukupova J, **Ticha I**, Janatova M, Sevcik J, Kleibl Z, Kleiblova P, Pohlreich P (2010) *Alterations in BRCA1, BRCA2, TP53 and ATM genes in sporadic breast tumours.* EJC Suppl. 8:203.

### 9.1.2 Author's awards

**Ticha I** (2009) *Dlouhé delece a přestavby v lokusu BRCA1 u pacientek s hereditárním syndromem karcinomu prsu a ovaria*. Winning oral presentation – theoretical postgraduate section, 10th Studen's Scientific Conference, First Faculty of Medicine, Charles Univerzity in Prague.

## 9.2 Publications in extenso not related to PhD Thesis

Kleiblová P, Dostalová I, Bártlová M, Lacinová Z, **Tichá I**, Krejčí V, Springer D, Kleibl Z, Haluzík M (2010) *Expression of adipokines and estrogen receptors in adipose tissue and placenta of patients with gestational diabetes mellitus.* Mol Cell Endocrinol. 314:150-156 (**$IF_{2008}$ = 3.611**)

### 9.2.1 Published abstracts

**Ticha I**, Maňásková P, Liberda J (2005) *Sow uterus enzymatic degradation and properties of boar cowper glands secretion.* Oral presentation – XIth Symposium of Czech Reproductive Immunologists with International Participation, The Castle, Zdar nad Sazavou 26-29 May 2005.

**Ticha I**, Maňásková P, Liberda J (2006) *Study of boar cowper gland secretion.* Oral presentation – XIIth Symposium of Czech Reproductive Immunologists with International Participation, The Castle, Zdar nad Sazavou 25-28 May 2006.