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New sequence variants in *BRCA1* and *BRCA2* genes detected by high-resolution melting analysis in an elderly healthy female population in Croatia

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Abstract

Background: Mutations in *BRCA1* and *BRCA2* genes are associated with family predisposition to breast and ovarian cancer. Novel screening methods are required for efficient and rapid detection of sequence variants in cancer patients and their family members.
Methods: The screening for variants in the breast and ovarian cancer susceptibility genes *BRCA1* and *BRCA2* in Croatia was performed by a high-resolution melting approach, which is based on differences in melting curves caused by variations in nucleotide sequence. This is the first screening in Croatia on elderly healthy women with no family history of cancer. *BRCA1* screening was performed on 220 and *BRCA2* screening on 115 samples.
Results: In a population well beyond the average age of breast/ovarian cancer onset, 21 different sequence variants in the *BRCA1* gene (one novel: *c.5193+49_50delTA*) and 36 variants in the *BRCA2* gene (7 novel: *c.459A>C*, *c.3318C>A*, *c.4412_4414delGAA*, *c.4790C>A*, *c.6264T>C*, *c.9087G>A*, and *c.9864A>G*) were detected.
Conclusions: Nine *BRCA1* and seven *BRCA2* known variants appeared with such high frequencies that they could be declared as harmless in this population. Eight *BRCA1* high frequency variants, located further from the promoter region, appear to be strongly correlated. Three novel variants that changed the amino acid sequence of the BRCA2 protein (two missense base substitutions, *c.3318C>A* and *c.4790C>A*, and one codon deletion *c.4412_4414delGAA*), appearing only once, were predicted to have no potential effect on protein structure and function.
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Keywords: allele frequency; *BRCA1*; *BRCA2*; high-resolution melting.

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Introduction

BRCA1 and *BRCA2* genes, involved in DNA repair processes, are the major breast and ovarian cancer susceptibility genes (1, 2). Epidemiological data indicate that 5%–10% of all breast and/or ovarian cancers are associated with inherited mutations in *BRCA* genes (3, 4). The penetrance of deleterious *BRCA* mutations has been variably estimated; a recent combined analysis of different reports (5) estimates that average cumulative risks (by age 70 years) in *BRCA1* mutation carriers are 65% for breast cancer and 39% for ovarian cancer, whereas the corresponding risks for *BRCA2* are 45% and 11%, respectively.

Carriers of *BRCA1* or *BRCA2* mutations are also at increased risk for other cancers: uterine, cervical, early-onset prostate and pancreatic cancer in *BRCA1*, and male breast, prostate, pancreatic, gallbladder, bile duct, stomach cancers and melanoma in *BRCA2* (6, 7).

Various *BRCA* mutations associated with breast/ovarian cancer may significantly differ in distribution; some were found to be unique to the family tested or to a specific group, whereas others appeared across different populations (8). Most reported disease-associated alleles of *BRCA1* and *BRCA2* have been attributed to frameshifts, nonsense or missense mutations, large rearrangements and splice alterations. They usually lead to the truncation of BRCA1 or BRCA2 protein, or affect amino acids that are critical for its structure or function. However, a large number of sequence variants, in particular missense variations, routinely encountered in clinical and research laboratories, cannot be readily distinguished as either disease-causing (deleterious) mutations or benign polymorphisms (clinically not significant), and thus are classified as variants of unknown clinical significance.

To date, more than 3500 *BRCA1* and *BRCA2* variants have been reported in the Breast Cancer Information Core (BIC) database (http://research.nhgri.nih.gov/projects/bic/index.shtml). This database comprises accumulated research data gained from both diseased and healthy individuals (9); no distinction is made between them, except for classification by clinical significance.

No data on *BRCA* variants in an affected or healthy population of Croatia have been collected so far. Therefore, a pilot project involving the screening of healthy women with the intention to facilitate the introduction of genetic testing into the national program of early detection of breast and ovarian cancer was undertaken. In the Croatian population of 4.5 mil-

lion, an average of 2200 new breast cancer and 400 ovarian cancer cases have been reported annually over the last 10 years (with a moderate tendency of increase), and 800 women die of breast cancer each year (data from the Central Bureau of Statistics of Republic of Croatia and Croatian National Institute of Public Health, 2006). Subjects for the screening pilot project were recruited among elderly women with no personal or family history of cancer, in order to identify benign high frequency variants of *BRCA1* and *BRCA2* in the Croatian population.

The screening was performed by a high-resolution melting approach, which is based on differences in melting curves caused by variations in nucleotide sequence; detected variants were confirmed by direct sequencing. The high-resolution melting approach is a rapid method for screening and detection of nucleotide variations.

Patients and methods

Initially, 115 healthy Croatian females, aged between 64 and 100 years (mean age 78±8; median 76 years) were selected to be screened for *BRCA1* (MIM#113705) and *BRCA2* (MIM#600185) sequence variants. Subsequently, the analysis of *BRCA1* was extended to another group of 105 healthy women (matched by age with the first group) for reasons described below.

All subjects were healthy, not undergoing any therapy, and with no family history of breast, ovarian, prostate, colon, pancreatic, or any other cancer. They were recruited from several locations in the Zagreb area (General Medical Practice Kalinovica, General Medical Practice Dobojska, Gynecological Clinic Srednjaci and Nursing Home for the Elderly Godan). Blood samples were taken from subjects after they had signed an informed consent form and were collected adhering to all necessary ethical and legal requirements; all were stripped of identifiers and could not be traced back to subjects. All patients gave their informed consent to perform DNA analysis on their blood samples before the samples were taken. The study was conducted according to the Declaration of Helsinki principles and approved by the Ethical Committee of Clinical Hospital Petrova, University School of Medicine, Zagreb (No. 021-1/49-2006) and by the Ethical Committee of Medical Center Zagreb-West based on Health Care Law of Republic of Croatia (NN121/03/). The study was also strongly supported by the Croatian League Against Cancer (http://www.hlpr.hr) and the Croatian Society of Human Genetics (http://hdhg.mef.hr).

DNA was extracted from peripheral blood leukocytes, and the entire coding sequence and exon-intron boundaries were amplified using polymerase chain reaction (PCR) resulting in 36 PCR products sized 150–437 bp for *BRCA1* and 49 PCR products 179–500 bp long for *BRCA2* (10).

PCR was performed in 10 μL reaction mixture containing 50 ng template DNA, 0.2 mM dNTPs (Roche, Mannheim, Germany), 0.4 U FastStart Taq DNA Polymerase (Roche), 1× fluorescent dye LCGreen Plus (Idaho Technology, Salt Lake City, ID, USA), 2 mM $MgCl_2$ (Idaho Technology) and forward and reverse primers (0.5 mM each) for each gene segment, in Roche LightCycler capillaries and amplified in an adapted RapidCycler2 instrument (Idaho Technology). PCRs were performed using appropriate sets of primers as described previously (10) with slight modifications (primer sequences are available by e-mail: levanat@irb.hr).

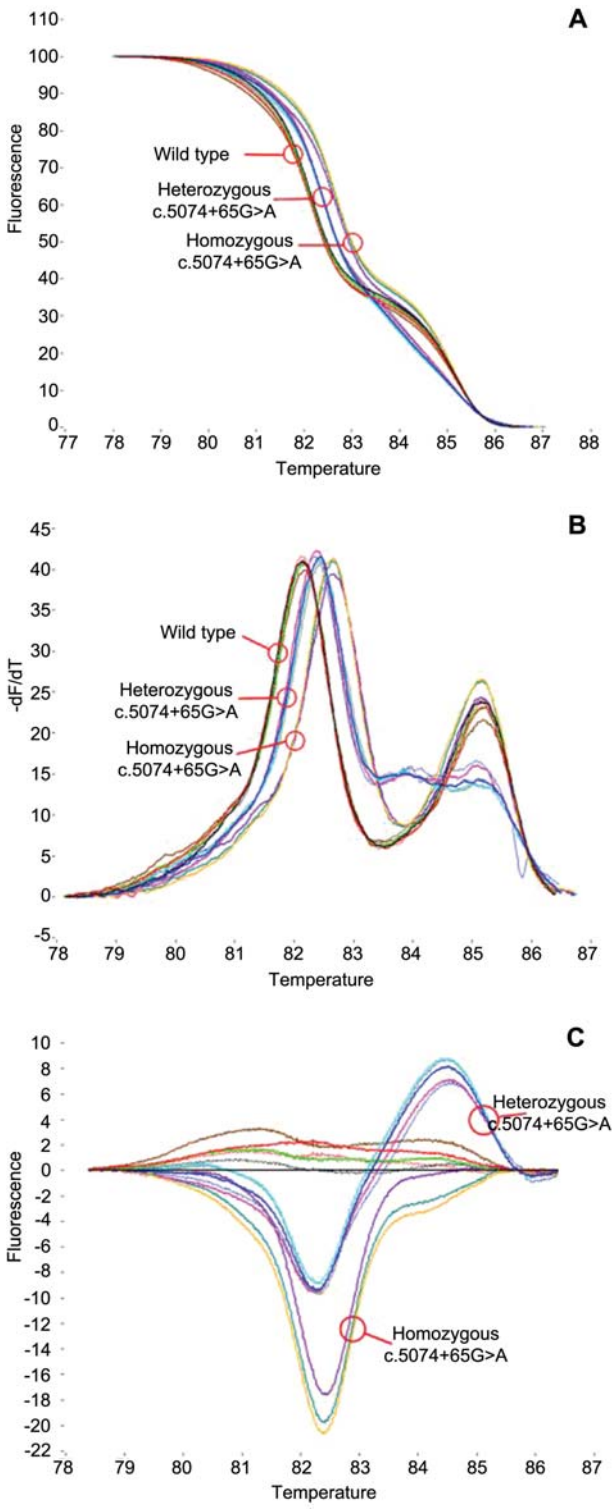

Figure 1 Melting profiles analyzed with HR-1 software. (A) Melting curve (plot of fluorescence vs. temperature) of several samples analyzed for exon 17 of the *BRCA1* gene. Three distinct groups of curves represent different genotypes: wild type, *c.5074+65G>A* homozygous, and *c.5074+65G>A* heterozygous. (B) Samples shown as a derivative plot (–dF/dT vs. temperature), showing the same three groups of samples. (C) Samples shown as difference plot (fluorescence vs. temperature), compared to a known wild type sequence.

PCR conditions were optimized to temperatures between 49°C and 68°C for each segment. After 40 cycles of amplification, PCR products underwent an additional 1 min at 98°C and then 5 min at 40°C to promote heteroduplex formation.

Each capillary was then transferred to the High-Resolution Melter instrument (HR-1, Idaho Technology) for high-resolution melting and curve analysis. Samples were melted at 0.2°C/s ramp rate. Melting profiles were analyzed with HR-1 software using fluorescence normalization, temperature shift and conversion to difference and derivative plots (Figure 1). Fragments with melting patterns different from the wild type were sequenced to determine the exact sequence alterations (11, 12). Coding variants are described according to the GenBank accession number U14680 for *BRCA1* and GenBank accession number NM_000059.3 for *BRCA2* reference sequences. Intronic variants are described according to GenBank accession number NG_005905.1 for *BRCA1* and GenBank accession number NW_001838072.1 for *BRCA2* genes. Nucleotide numbering is based on cDNA sequence and nucleotide +1 corresponds to A of the ATG translation initiation codon. The nomenclature used in this study follows the Nomenclature for Description of Genetic Variations approved by the Human Genome Variation Society.

Before sequencing, the chosen PCR products were purified with ExoSAP-IT (USB, Cleveland, OH, USA) and then sequenced in both directions using the Big Dye Terminator 1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequencing analysis was performed on an automatic sequencer ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Associations among nine *BRCA1* sequence variants with highest minor allele frequency (all conformed to Hardy-Weinberg equilibrium) were assessed by measuring pairwise linkage disequilibrium (LD) using r^2 statistics. All calculations were carried out using HaploView (13).

Online splice prediction tools NNSPLICE (http://www.fruitfly.org/seq_tools/splice.html) (14), SpliceSiteFinder (http://violin.genet.sickkids.on.ca/~ali/splicesitefinder.html), and ESEfinder3.0 (http://rulai.cshl.edu/tools/ESE/) (15) were used to examine potential impact on the splicing for the newly found variants. We used increased threshold values of 2.0 for SF2/ASF and 3.0 for SC35, SRp40, and SRp55 motives in order to reduce false-positive results (16).

Results

Detected variants of *BRCA1* and their frequencies in the enlarged group of 220 subjects are listed in Table 1. The results for *BRCA2* in the initial group of 115 subjects are listed in Table 2. Novel variants are given in boldface.

BRCA1

We detected 21 different sequence variants in the *BRCA1* gene: 11 variants were missense, four were synonymous and six were intronic. Figure 2 shows their distribution along the gene/protein and the proportion of homozygous/heterozygous carriers.

One novel sequence variant was detected: the intronic variant *c.5193+49_50delTA*. For this intronic variant, it was predicted by both NNSPLICE and SpliceSiteFinder that a deletion of two nucleotides would not change the score probability for the recognition of either donor or acceptor site.

Screening for *BRCA1* variants was performed in the enlarged group, because the results for the initial 115 subjects were suggesting strong correlation between several high frequency variants. In order to obtain a statistically more reliable picture, an additional 105 women were screened.

In the enlarged population, only four additional low frequency variants were detected (they are included

Table 1 *BRCA1* sequence variants in 220 healthy Croatian women (novel variant in boldface).

Nucleotide change	AA change	Proportion of carriers, %	Allele frequency, %	Exon	BIC accession no. or reference	Times reported in BIC	Clinically important (reference)
Missense variants (n=11)							
c.3548A>G	p.Lys1183Arg	49.09	27.50	11	1099	33	No
c.4837A>G	p.Ser1613Gly	46.82	24.77	16	1140	36	No
c.3113A>G	p.Glu1038Gly	46.36	24.55	11	1087	37	No
c.2612C>T	p.Pro871Leu	43.18	22.05	11	1067	26	No
c.1067A>G	p.Gln356Arg	11.36	5.68	11	2558	82	No/little (17)
c.3119G>A	p.Ser1040Asn	4.09	2.05	11	1089	45	No/little (17)
c.4956G>A	p.Met1652Ile	2.73	1.36	16	1143	39	No/little (17)
c.4535G>T	p.Ser1512Ile	1.36	0.68	15	1136	53	No
c.2077G>A	p.Asp693Asn	0.91	0.45	11	1045	16	No
c.4039A>G	p.Arg1347Gly	0.91	0.45	11	1351	154	No/little (17)
c.2002C>T	p.Leu668Phe	0.45	0.23	11	2313	25	Unknown
Synonymous variants (n=4)							
c.4308T>C	p.=	42.27	25.23	13	1128	35	No
c.2082C>T	p.=	44.55	24.77	11	1047	14	No
c.2311T>C	p.=	40.00	20.23	11	1055	25	No
c.1911T>C	p.=	0.45	0.23	11	Judkins et al. (18)	0	No (18)
Intronic variants (n=6)							
c.5074+65G>A		45.91	26.59	17	Song et al. (19)		No (19)
c.442-34C>T		43.18	22.95	8	1424	9	No
c.5075-53C>T		0.91	0.45	18	10455	2	Unknown
c.5277+48_59dup12		0.91	0.45	20	1292	26	No (20)
c.301+7G>A		0.45	0.23	6	2238	12	Unknown
c.5193+49_50delTA		**0.45**	**0.23**	**19**	**–**	**0**	

Table 2 *BRCA2* sequence variants in 115 healthy Croatian women (novel variants in boldface).

Nucleotide change	AA change	Proportion of carriers, %	Allele frequency, %	Exon	BIC accession no. or reference	Times reported in BIC	Clinically important (reference)
Missense variants (n = 13)							
c.1114A>C	p.Asn372His	35.65	19.13	10	1668	9	No
c.865A>C	p.Asn289His	8.70	4.35	10	1129	13	No
c.2971A>G	p.Asn991Asp	8.70	4.35	11	1903	6	No
c.5744T>C	p.Met1915Thr	5.22	2.61	11	1108	7	Unknown
c.968C>A	p.Ser326Arg	3.48	1.74	10	1222	105	No
c.3515C>T	p.Ser1172Leu	2.61	1.30	11	1908	43	No (21)
c.7544C>T	p.Thr2515Ile	2.61	1.30	15	1547	71	No
c.125A>G	p.Tyr42Cys	0.87	0.43	3	1004	141	No (22)
c.3318C>A	**p.Ser1106Arg**	**0.87**	**0.43**	**11**	**–**	**0**	
c.4258G>T	p.Asp1420Tyr	0.87	0.43	11	1274	191	No
c.4790C>A	**p.Ser1597Tyr**	**0.87**	**0.43**	**11**	**–**	**0**	
c.6100C>T	p.Arg2034Cys	0.87	0.43	11	1324	97	Unknown
c.6935A>T	p.Asp2312Val	0.87	0.43	12	5423	5	No (21)
Synonymous variants (n = 14)							
c.3396A>G	p.=	46.09	23.91	11	1661	8	No
c.7242A>G	p.=	43.48	23.04	14	1125	10	No
c.3807T>C	p.=	33.04	19.57	11	1662	3	No
c.1365A>G	p.=	8.70	4.35	10	1106	7	No
c.2229T>C	p.=	7.83	3.91	11	13616	7	No
c.6264T>C	p.=	**1.74**	**0.87**	**11**	**–**	**0**	
c.198A>G	p.=	0.87	0.43	3	Konstantopoulou et al. (23)		No (23)
c.459A>C	p.=	**0.87**	**0.43**	**5**	**–**	**0**	
c.1938C>T	p.=	0.87	0.43	11	2907	2	No
c.3516G>A	p.=	0.87	0.43	11	3501	4	No
c.4068G>A	p.=	0.87	0.43	11	3525	3	Unknown
c.5199C>T	p.=	0.87	0.43	11	1941	2	No
c.9087G>A	p.=	**0.87**	**0.43**	**23**	**–**	**0**	
c.9864A>G	p.=	**0.87**	**0.43**	**27**	**–**	**0**	
Intronic and non-coding variants (n = 5)							
c.7806-14C>T		73.04	46.52	17	1126	15	No
c.6841+80_83delTTAA		61.74	38.26	11	2022	1	Unknown
c.-26G>A		55.65	35.65	2	1666	12	No
c.425+67A>C		8.70	4.35	4	Seo et al. (24)		No (24)
c.9257-16T>C		0.87	0.43	25	1123	6	Unknown
Truncating mutations (n = 3)							
c.5645C>A	p.Ser1882X	0.87	0.43	11	1036	26	Yes
c.9976A>T	p.Lys3326X	0.87	0.43	27	1179	293	No
c.10095delCins11	p.Ser3366AsnfsX4	0.87	0.43	27	1788	11	No
Deletions (n = 1)							
c.4412_4414delGAA	***p.Arg1471del***	**0.87**	**0.43**	**11**	**–**	**0**	

among the 21 variants in Table 1). Also, the previous structure of the high frequency group (nine variants with similar allele frequencies, 20%–30% each) remained essentially unchanged (online supplementary Figure 1 illustrates the frequencies for the entire set of subjects). Characterization of linkage disequilibrium among nine most frequent *BRCA1* (Table 1) sequence variants proved that eight out of nine of them manifest higher association (r^2 from 0.346 to 0.768), while sequence variant *c.442-34C>T* stays clearly outside that cluster (Figure 3).

BRCA2

We found 36 different sequence variants in the *BRCA2* gene: 13 of them were missense, 14 synonymous, five intronic variants, three truncating mutations and one deletion. Figure 4 shows their distribution along the gene/protein and the proportion of homozygous/heterozygous carriers.

Seven novel sequence variants were detected: four synonymous (*c.459A>C*, *c.6264T>C*, *c.9087G>A*, and *c.9864A>G*), two missense (*c.3318C>A* and *c.4790C>A*) and one deletion (*c.4412_4414delGAA*). For the synonymous variant *c.459A>C* in exon 5 (N-terminal), ESEfinder 3.0 predicted loss of one SRp40 motif and appearance of one SF2/ASF motif. For the *c.6264T>C* in exon 11 (BRC repeats), it predicted loss of one SF2/ASF motif and gain of one SC35 motif. The other two novel synonymous variants, both missense variants and the deletion, analyzed in silico, do not cause changes in ESE splicing motifs. None of these variants leads to the creation of potential cryptic splice sites.

Variants of *BRCA2* were also divided into two frequency groups (online supplementary Figure 2), but

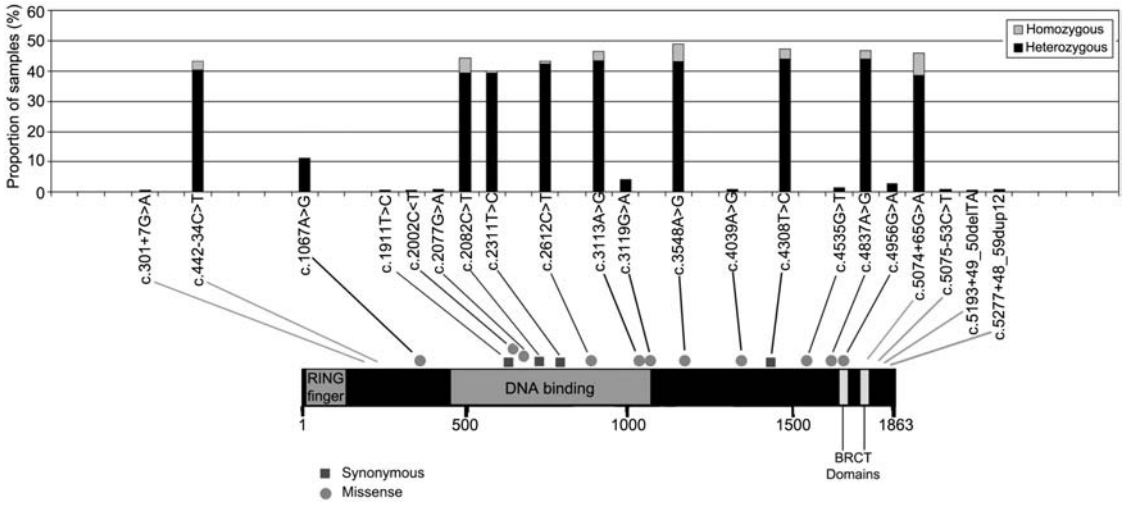

Figure 2 Locations of 21 detected BRCA1 sequence variants in relation to BRCA1 protein with its domains.
Circles represent missense variants, squares synonymous, whereas non-coding variants are drawn only with gray lines denoting their position relative to coded counterparts. Columns above the protein represent the proportion of heterozygous (black) and homozygous (gray) carriers for each variant.

with great variations within the high frequency group, so the screening was not extended beyond the initial set of 115 women. Three leading variants appeared with far greater frequencies than in the *BRCA1* case and differed too much among themselves as well as from the others for any large scale correlation.

Discussion

Distribution of variants across the protein domains

BRCA1 protein contains two highly conserved regions; one is the RING finger domain and the other BRCT domains (Figure 2). Mutations in the RING finger domain were detected in breast cancer patients from Chile, Japan and India (25–27), and several large studies in Italy and Germany (6, 28–37) showed polymorphisms and mutations in this region in patients but also in the control population. However, consistent with the conserved status of the region, we did not detect any variants of the RING finger domain in the healthy Croatian population. The only alteration we found in the BRCT domains that could alter DNA repair function was *c.4956G>A*, but it had already been classified as a neutral polymorphism (38–40).

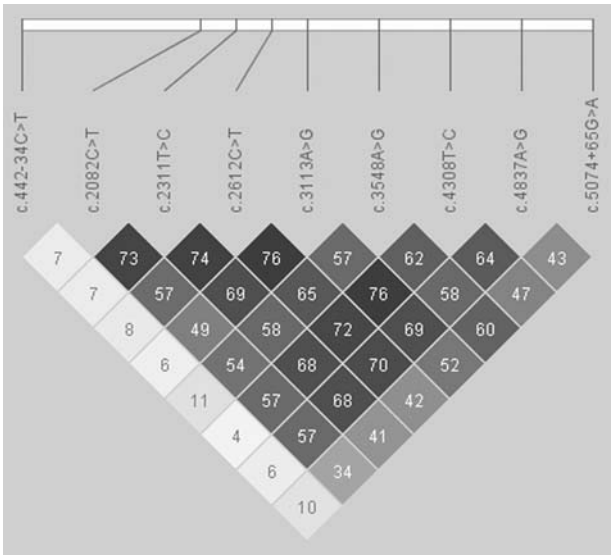

Figure 3 Pairwise linkage disequilibrium (LD) of nine *BRCA1* sequence variants with highest minor allele frequency.
The location of each sequence variant along the *BRCA1* gene is relative to the real nucleotide position. The number in each diamond indicates the intensity of LD ($r^2 \times 10^{-2}$) between respective pairs of sequence variants. The strength of LD is represented by shades of gray (0 [white] $< r^2 <$ 1 [black]).

Most of the *BRCA1* variants detected in the Croatian population were in the DNA binding domain and downstream. High incidence of missense variants indicates this region is not as vital as the RING finger or BRCT domains. These missense variants have previously been reported in both healthy individuals and patients with similar frequencies, indicating that they are not clinically significant (27, 30, 38–40).

Unlike *BRCA1*, *BRCA2* conserved regions were not so free of variants. The conserved BRC repeats region (Figure 4) is essential for the BRCA2 protein function in DNA repair and contains the ovarian cancer cluster region (OCCR, bounded by nucleotides 3059–4075 and 6503–6629) that is associated with ovarian cancer family history (41). Mutations in the BRC repeats are common in Japanese, Indian, German, Greek and Korean breast and ovarian cancer patients (26, 27, 36, 42–44). However, in this region we found 15 different variants in the healthy Croatian population, although most of them with low incidence (the exceptions were two synonymous variants). In the other conserved region of the *BRCA2* gene, the C-terminal domain, we detected only four variants. One of them was synonymous, two non-coding, and one missense variant (*c.7544C>T*), which had been previously reported to have no effect on the *BRCA2* protein function (45).

Remaining parts of the *BRCA2* sequence are not as conserved. Here, we detected most variants, includ-

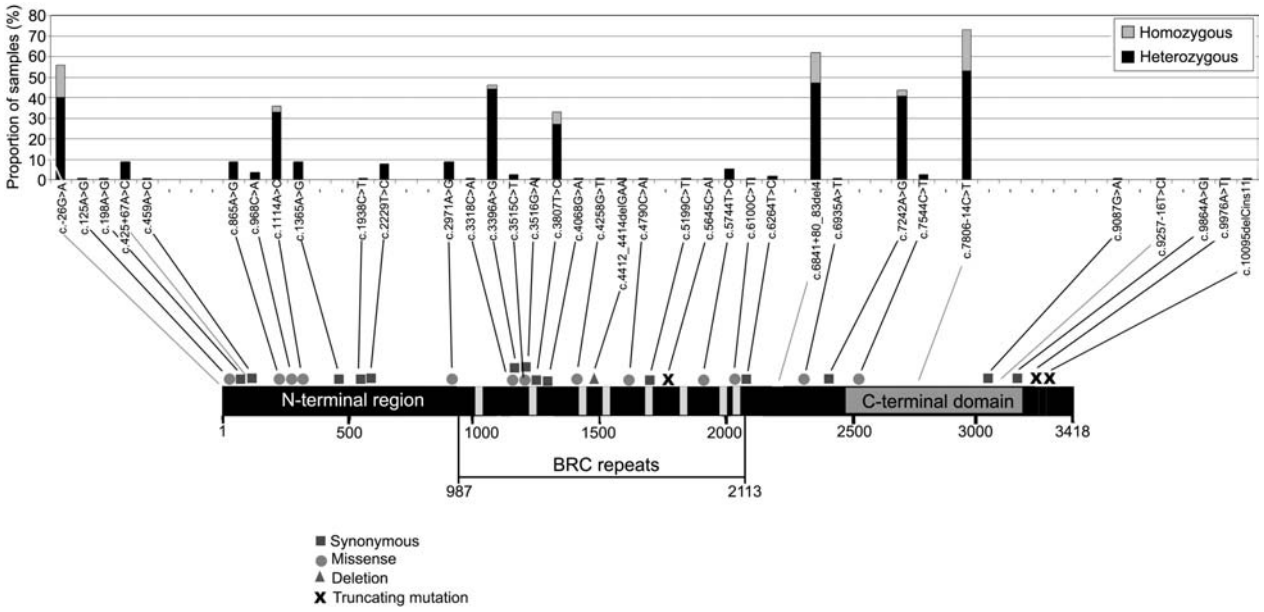

Figure 4 Locations of 36 detected *BRCA2* sequence variants in relation to *BRCA2* protein with its domains.
Circles represent missense variants, squares synonymous, triangle is a deletion and X denotes truncating mutations, whereas non-coding variants are drawn only with gray lines denoting their position relative to coded counterparts. Columns above the protein represent the proportion of heterozygous (black) and homozygous (gray) carriers for each variant.

ing five missense alterations, as well as two stop variants leading to truncation of the C-terminus of the protein.

Clinical significance of the variants

The elderly population in this screening was well beyond the average age of onset of breast/ovarian cancer; therefore, high frequency variants of *BRCA1* and *BRCA2* would be most unlikely to cause cancer predisposition. Due to the sharp division between high and low frequency groups, a conservative limit of more than 30% carriers can be set for both genes, leading to nine *BRCA1* and seven *BRCA2* variants that can be declared as harmless in this population. All these high frequency variants also regularly appear in homozygous form, indicating them as harmless, since homozygous mutations in *BRCA1* are usually lethal (46) and in *BRCA2* lead to Fanconi anemia (47) (OMIM 605724).

Significance of the lower frequency variants cannot be judged on the basis of our screening: 12 of them appeared in 2%–11% of the subjects and the remaining 29 were of the order of 1% or less. Their very low appearance among these elderly subjects with no family history of cancer does not support their predisposing potential, or at least suggests limited penetrance. Only one variant from our population, *c.5645C>A* in *BRCA2*, has been classified as deleterious mutation (29). The German Consortium for Hereditary Breast and Ovarian Cancer found this mutation in two different families (33). We found it in a single sample and in a heterozygous form (an 87-year-old woman with no family history of breast or ovarian tumors, and with no sporadic disease). We cannot speculate any more on the significance of this variant in tumor formation. Various factors play a role in tumor formation and a mutation is only one of them. A mutation in one of these genes does not imply the person will develop a tumor; it only increases the risk, which can be as high as 80% by the age of 70 (8). Samples with a family history of cancer may provide answers about penetrance and expressivity of the complex cancer disease.

All our novel variants appear in one or two cases each, so their significance cannot be judged by incidence. For missense variants *c.3318C>A* and *c.4790C>A*, tools, such as Align-GVGD (http://agvgd.iarc.fr) (38), SIFT (http://blocks.fhcrc.org/sift/SIFT.html) (48), and PolyPhen (http://coot.embl.de/PolyPhen/) (49), have predicted that these amino acid changes in *BRCA2* protein are tolerated so they could have no potential effects on protein function. Although the 3D structure of complete *BRCA2* is not yet determined, arginine 1471 in *BRCA2* protein is not conserved among species (38), neither does it belong to any functional domain (http://smart.embl-heidelberg.de) (50). Therefore, it is likely that deletion of amino acid at that position also has no potential harmful effects. The two synonymous variants (*c.459A>C* and *c.6264T>C*) showed potential changes in splicing motifs, which could disrupt the usual splicing pattern. It has been previously reported that changes in exon sequence can cause exon skipping in *BRCA1* and *BRCA2* genes, although only for nonsense or missense mutations (51, 52). It was shown by Pettigrew et al. (53) that reported unclassified sequence variants in *BRCA2* were found to colocalize to 55% of predicted ESEs, while previously reported polymorphisms do not colocalize to the conserved ESEs. This suggests that potential motifs can be indicative if detected in unclassified variants, because they are not present in benign polymorphisms.

Application of high-resolution melting

Sensitivity and specificity of this method in scanning for polymorphisms have been previously shown to be 95% and 99%, respectively (11). In this study, we conducted sequencing of suspect forms, and all heterozygous forms were identified without error. Difference plot was the most informative in our study; different sequence variants were most easily distinguishable in this plot. All the variants were easily detected based on curve shapes, and the same type variants always grouped together (Figure 1); therefore, such variants could be easily distinguished without the need for sequencing. Samples with other sequence variants regularly differed from all other curves, clearly distinguishing them as different. All of these curve patterns were verified by sequencing. We tested the application of this method in mutation detection, using 25 coded samples with known mutations and detected them by high-resolution melting in all cases. All the curves differed from wild type and from all other variants (example in online supplementary Figure 3). High-resolution melting is an effective method for variant detection in *BRCA1* and *BRCA2* genes and can be applied for rapid screening of samples in gene testing (54, 55).

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