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Establishing a control population to screen for the occurrence of nineteen unclassified variants in the BRCA1 gene by denaturing high-performance liquid chromatography

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Abstract

Numerous missense mutations in BRCA1 and BRCA2 are detected during clinical screening of breast and ovarian cancer patients. Because of the lack of a functional protein assay to determine the functional consequence of these mutations, patients are often frustrated by inconclusive results due to unclassified variants (UV). To determine whether a reported UV is also present in a control collective and therefore more likely be a rare polymorphism than a deleterious mutation, we collected a control population consisting of 95 females and 25 males aged over 60 years (mean 73 years) without a family history of BRCA associated cancers. The age of the control group is beyond the median onset of breast and ovarian cancer with a hereditary background. These controls were analysed for the presence of 19 known UVs in BRCA1 with the DHPLC technique. Only four of the 19 variants (R496H, R866C, S1040N and M1652I) were detected and can be considered polymorphims. However, no firm conclusion can be drawn about the functional relevance of the other 15 variants.

Keywords: Denaturing HPLC; Unclassified variants; BRCA1 gene

1. Introduction

Since the detection of BRCA1 [1] and BRCA2 [2] several studies using families with multiple breast and/or ovarian cancers in various countries were performed. Although deleterious germline mutations in BRCA1 and BRCA2 confer a high risk of breast and ovarian cancer, even in family situations with a high a priori probability of hereditary breast cancer mutations, the mutation detection rate is lower than expected. As deleterious germline mutations in BRCA1 and BRCA2 confer a greatly increased risk of breast cancer, common variants in both genes could be ideal candidates for low penetrance alleles [3]. As more individuals are tested, and as testing is extended to more ethnically diverse and clinically representative populations, it is likely that the number of individuals found to carry such variants will also increase.

Because there are up to now no functional protein assays to determine the clinical consequence of the numerous BRCA1 and BRCA2 missense mutations identified during screening, patients are often frustrated by inconclusive results due to unclassified variants (UV). The temptation to implicate a missense mutation that occurs at low frequency as a

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potential functional lesion must be tempered by the possibility that the variant may exist more frequently in another population and thus be considered mere polymorphism. As example, a sequence variation with extremely rare occurrence among one population, though more frequent in another population, will not turn up in the control population either [4].

The median age of women at the time of diagnosis of breast cancer is 63.5 years and ovarian cancer 61 years. In a hereditary situation in most of the cases the age at the time of diagnosis will be before 50 years. The aim of the study was to collect a control population which is older than the average age of onset of breast/ovarian cancer and without family history of BRCA associate cancers. If in this population a UV is found, not only due to the blank family history but also of the age it could be assumed that this UV represents a rare polymorphism or at least an alteration with very low penetrance.

For the analysis of the samples we performed denaturing high-performance liquid chromatography (DHPLC), which has shown in several publications that it represents a highly sensitive, rapid and reproducible screening technology for unknown and know mutations [5]. The 19 UVs of the BRCA1 gene were chosen due to their occurrence in members of German hereditary breast and ovarian cancer families (HBOC) [6].

2. Experimental

2.1. Patients

Samples were obtained from 125 apparently unrelated individuals (95 females and 30 males) who had no BRCA1 and BRCA2 related carcinomas by her- and himself and in their family history. All patients gave informed consent using a standardised written form. DNA from blood samples were isolated using standard procedures. Mutational screening was performed by DHPLC and by dye terminator sequencing to confirm the alteration.

2.2. PCR and DHPLC

Published primer pairs and conditions [7,8] were used to amplify the BRCA1 fragments comprising the 19 variants (Table 1). To promote heteroduplex formation the PCR product was heated to 95 °C for 3 min and then cooled to 65 °C over 30 min. and stored at 4 °C until use.

DHPLC analysis was performed on a Wave DNA fragment analysis system (Transgenomic, San Jose, CA, USA) as previously reported [8]. The melting profile for each DNA fragment, the respective elution profiles and column temperature were determined using either the DNA melt software, which is available at http://insertion.stanford.edu/melt.html and described in [9], or WAVE maker software from Transgenomic. Heterozygous control probes were used to confirm system performance in general and the accuracy of the oven temperature in particular. All fragments which displayed a single peak in a first DHPLC run were analysed in a second run to detect possible homozygous alterations. In these cases, equal amounts of a PCR product from the same BRCA1 fragment of a known wild type sample were added to the test sample prior to the reannealing step to enable heteroduplex resolution.

The genotype of an alteration detected by DHPLC was predicted by comparison with profiles of the unclassified variants under investigation (standards). This was accomplished by superimposing chromatograms and assigning sample chromatograms to chromatograms of the UVs. Special care was taken to notice even smallest deviations between the sample peak pattern and the control peak, because the latter could indicate an unknown sequence alteration. Predictions were confirmed by sequence analysis. Sequencing was performed using BigDyeDeoxy terminator cycle sequencing reagents (Perkin-Elmer, Weiterstadt, Germany) as described previously [7].

2.3. Comparison with BRCA1 orthologs

For the alignment of the sequences comprising the investigated UVs with the sequence of other species submitted sequences in GenBank were used (dog: accession no. U50709 and chicken accession no. AF355273).

3. Results

Participants in the study were interviewed to determine their personal and family history of cancer. Those with a family history, especially car-

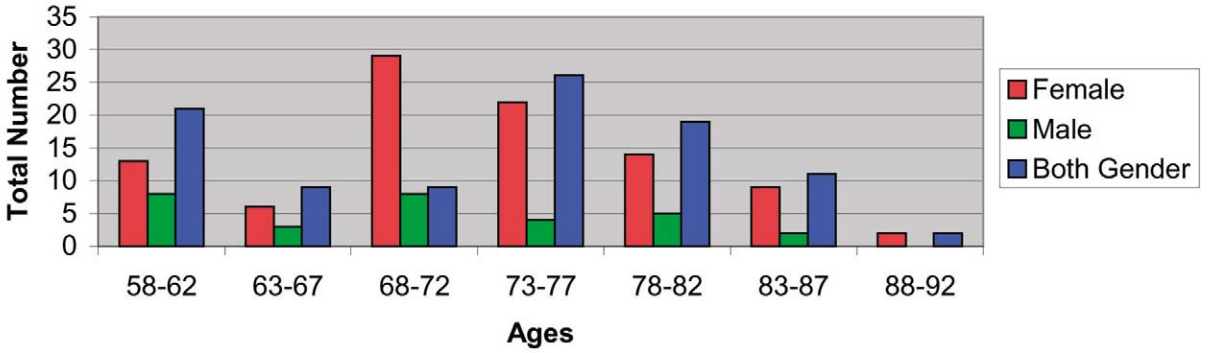

Fig. 1. Age distribution of the control population.

cinomas related to BRCA1 and BRCA2 mutations [10], were excluded and 125 blood samples could be collected. Due to the occurrence of male breast cancer in conjunction with BRCA2 mutations blood samples from 25 males were also included. As shown in Fig. 1 the ages for the females range from 58 to 92 years (median 73 years) and for the males from 59 to 87 years (median 70 years).

Nineteen missense alterations reported as UVs to the Breast Cancer Information Core (BIC) database (http: // www . nhgri . nih . gov / Intramural _ research / Lab_transfer / Bic /) and also occurring in the German HBOC families were analysed (Table 1). Out of them only four (R496H; R866C; S1040N and M1652I) could be found in our control sample. As shown in Table 1 the UV with the highest allele frequency in our control population were M1652I which were also reported together with a deleterious mutation in BIC database. Interestingly most of the individuals were the Uvs could be detected also

Table 1
Analysed UVs in the BRCA1 gene

Exon	Nucleotide change	Effect	BIC entry	Found in control population	Allele frequencies	Additional notes in BIC-database
2	c.129T>C	S4P	0	0×	0	
6	c.388T>C	I90T	1×[a]	0×	0	
8	c.628G>A	R170Q	1×	0×	0	
11	c.835A>G	H239R	1×[a]	0×	0	
11	c.1605C>T	R496C	10×	0×	0	
11	c.1606G>A	R496H	12×	2×	0.8	
11	c.2531G>C	Q804H	2×	0×	0	Not found in 50 controls (C. Mathew)
11	c.2596C>A	T826K	4×	0×	0	
11	c.2715C>T	R866C	8×	1×	0.4	
11	c.3238G>A	S1040N	20×	3×	1.2	
11	c.4071A>G	I1318V	0×	0×	0	
11	c.4158A>G	R1347G	107×	0×	0	Not found in 270 controls (A. Ostrander)
15	c.4654G>T	S1512I	50×	0×	0	
16	c5074T>C	M1652T	2×	0×	0	
16	c.5075G>A	M1652I	23×	6×	2.4	1× together with frameshift
17	c.5172A>G	T1685A	0×	0×	0	
18	c.5214C>T	R1699W	5×	0×	0	
23	c.5548T>G	V1810G	1×[a]	0×	0	Not found in 50 controls
24	c.5628T>C	W1837R	3×	0×	0	

[a] Reported by a German group.

inherited the BRCA1 haplotype 356Q/871L/1038G/1613G which occurred at a frequency of 0.32 [3] (Table 2). An overview of the probands record in correlation with the detected UVs is also shown in Table 2. In Fig. 2 an example of a DHPLC chromatogram with the respective sequence alteration is shown.

By sequence comparison, the M1652I variation was not only found in our control population but also as the wildtype constitution in the BRCA1 orthologues of the chicken. 496H also found in our controls represents the same in dog.

Demonstrating the power of the DHPLC technique we also detected two novel silent mutations c.1736G>A and c.4931A>G. The four different UVs found twelve times in the control population were predicted correctly by superimposing the specific DHPLC chromatograms. The known polymorphisms in the investigated fragments were also identified properly by the DHPLC chromatograms, reducing the sequencing efforts to a minimum.

4. Discussion

In the absence of widely available functional tests, possible strategies to clarify the significance of genetic variants are (a) tracking of the variant with breast and ovarian cancer in several families, (b) case–control studies, (c) occurrence in known orthologues and (d) characterization of the remaining allele for either presence or absence of the UV in several tumor samples. For example, if a variant does not track with the breast and ovarian cancers in several families, or if it is seen in conjunction with known deleterious mutations, it may be inferred that the variant is less likely to be of clinical significance. Further, variants may be classified as benign poly-

Table 2
Correlation of detected UVs in the BRCA1 gene with proband data

Gender	Age (years)	Exon	Nucleotid change	Effect	Tumors found in the proband	Tumors found in the family
Female	73	16	c.4956A>G; c.5075G>A	S1613G; M1652I	0	0
Female	77	16	c.4956A>G; c.5075G>A	S1613G; M1652I	0	0
Female	62	16	c.4956A>G; c.5075G>A	S1613G; M1652I	Cervix-Ca;	0
Female	73	16	c.4956A>G; c.5075G>A	S1613G; M1652I	Corpus-Ca	0
Male	78	16	c.4956A>G; c.5075G>A	S1613G; M1652I	0x	0
Male	63	16	c.4956A>G homozygous; c.5075G>A	S1613G; M1652I	0×	0
Female	59	11	c.3238G>A	S1040N	0	Brain tumor, Melanoma
Male	59	11	c.3232A>G; c.3238G>A	E1038G; S1040N	0	0
Male	69	11	c.3232A>G; c.3238G>A	E1038G; S1040N	Lung-Ca	0
Female	71	11	c.1606G>A	R496H	Colon-Ca	0
Female	75	11	c.1606G>A	R496H	Vulva-Ca	Colon-Ca; Testis-Ca
Female	82	11	c.2430T>C; c.2731C>T; c.2715C>T	L771L; P871L; R866C	0	0

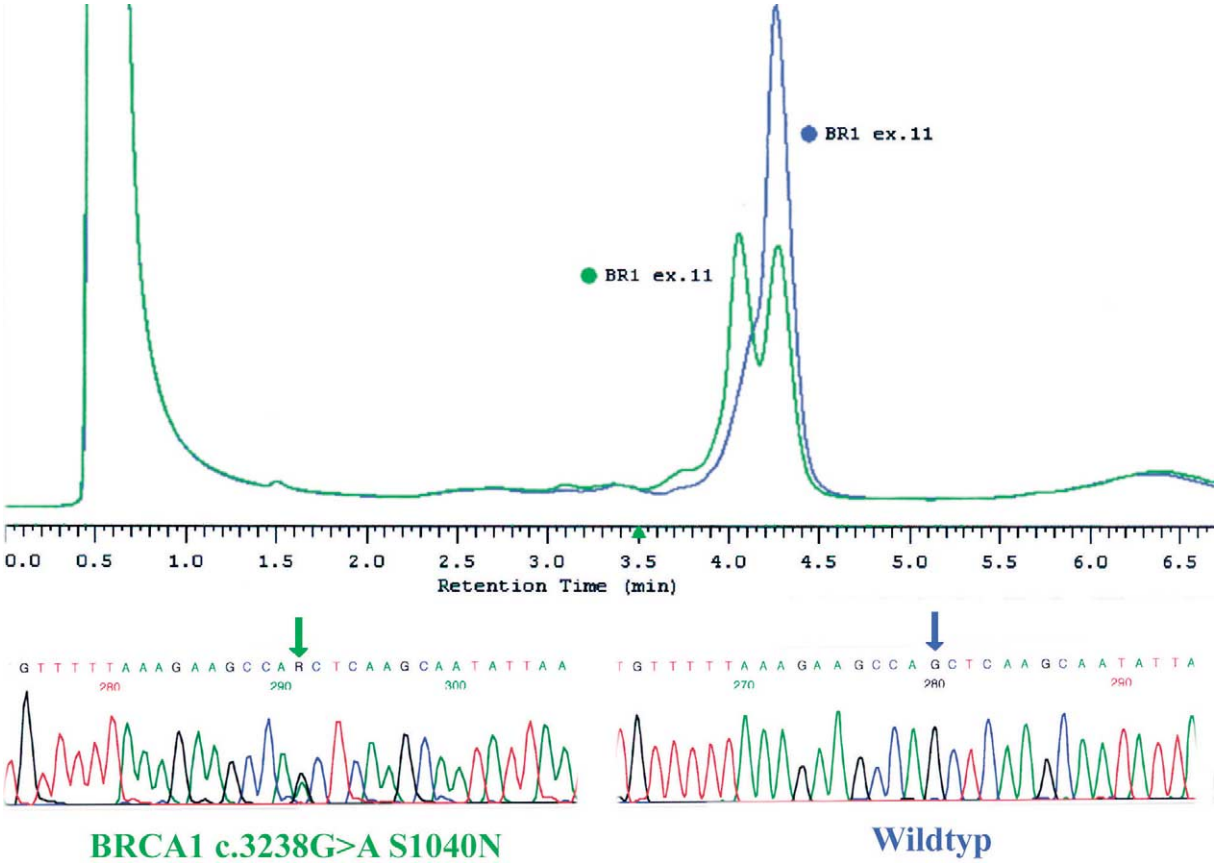

Fig. 2. DHPLC elution pattern of heterozygous sequence alteration in BRCA1 exon 11. Upper part displays the overlay of chromatograms showing BRCA1 c.3238G>A sequence alteration (green, heterozygous) and wildtype (blue). In the lower part are the respective sequences (arrows in the same color as the corresponding chromatogram mark the region of interest).

morphisms if they are observed in control samples, but it is difficult to know what frequency in the controls is sufficient to make that judgement. But nevertheless large population-based screening studies are needed to establish the frequency, importance, and penetrance of the broad spectrum of variations in the sequence of BRCA1 and BRCA2 observed in both affected and unaffected woman in the general population. Only then will it be possible to offer reliable results and meaningful counselling to women who choose to have a genetic test.

A control group consisting not only of individuals with no family history of tumors in conjunction with the gene in investigation but also of an age over 60 years has the advantage that if a UV is found in only a rare frequency it should be of low penetrance if it is disease associated.

In the up to now biggest case–control study in BRCA1 [11] the S1040N variant was also found in the control population. Together with our finding making it now more clear that this represents a benign polymorphism. On the other hand, the most frequently reported variant R1347G (107 times in the BIC database) was not found in our controls or in another study [11]. However, Langston et al. [12] found this variant in one person of their control group and in another individual together with a frame shift mutation, suggesting that the alteration represents a rare neutral polymorphism rather than a true mutation.

Information from interspecific alignments can indicate amino acid residues in gene products that are likely to produce disease if mutated in humans. Likewise, some positions in protein sequences vary among species, and such variable sites may indicate positions that are under less severe selective constraints. These variable positions suggest sites where residue changes can be tolerated by natural selection and provide insights into the types of amino acids that can be freely exchanged without negatively impacting protein function [13]. The UV M1652I fulfil all criteria mentioned to support the evidence for a benign polymorphism. The UV was found in our control population, reported together with a deleterious mutation in the BIC database and interspecific alignment showed that 1652I represents the wildtype situation in other species.

Besides the four variants found in our controls, which can be considered polymorphisms, no firm conclusion can be drawn about the functional relevance of the other 15 variants.

In the present study and other reports [6] it also could be shown that DHPLC fulfils the technical requirements to investigate several alterations in bigger sample sizes not only cost effective but also in a reasonable time. Amplimers containing known polymorphism with multiple alleles can markedly increase the sequencing cost for screening a large population. The known variants investigated in the study performed specific chromatogram profiles different from the known polymorphisms also occurring in the fragments in a specific number. Therefore, sequencing was unnecessary for DHPLC chromatograms characteristic for the specific polymorphisms.

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