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PRECLINICAL STUDY

Large *BRCA1* and *BRCA2* genomic rearrangements in Danish high risk breast-ovarian cancer families

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Abstract *BRCA1 and BRCA2* germ-line mutations predispose to breast and ovarian cancer. Large genomic rearrangements of *BRCA1* account for 0–36% of all disease causing mutations in various populations, while large genomic rearrangements in *BRCA2* are more rare. We examined 642 East Danish breast and/or ovarian cancer patients in whom a deleterious mutation in *BRCA1* and *BRCA2* was not detected by sequencing using the multiplex ligation-dependent probe amplification (MLPA) assay. We identified 15 patients with 7 different genomic rearrangements, including a *BRCA1* exon 5–7 deletion with a novel breakpoint, a *BRCA1* exon 13 duplication, a *BRCA1* exon 17–19 deletion, a *BRCA1* exon 3–16 deletion, and a *BRCA2* exon 20 deletion with a novel breakpoint as well as two novel *BRCA1* exon 17–18 and *BRCA1* exon 19 deletions. The large rearrangements in *BRCA1* and *BRCA2* accounted for 9.2% (15/163) of all *BRCA1* and *BRCA2* mutations in East Denmark. Nine patients had the exon 3–16 deletion in *BRCA1*. By SNP analysis we find that the patients share a 5 Mb fragment of chromosome 17, including *BRCA1*, indicating that the exon 3–16 deletion represents a Danish founder mutation.

Keywords *BRCA1* · *BRCA2* · Breakpoint · Danish founder mutation · Deletion · Duplication · MLPA · SNP array

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Introduction

Germ-line *BRCA1* (MIM# 113705) and *BRCA2* (MIM# 600185) mutations confer a lifetime risk of approximately 80% for breast cancer and 20–50% for ovarian cancer in female carriers, and in addition a moderate increased risk of other cancer types [1]. The *BRCA1* locus was linked to chromosome 17q21 in 1990 [2] and cloned in 1994 [3]. The gene spans approximately 81 kb and is composed of 23 exons encoding a protein of 1863 amino acids. Sequence conservation between mammalian species is weak, but a Zinc-binding RING domain at the amino terminus, involved in BARD1 binding [4], and an acidic carboxyl terminus containing two BRCA1 C-terminal (BRCT) domains, involved in protein interactions with several proteins including CtIP, BRIP1, and Abraxas [5–7], are highly conserved. The *BRCA2* locus was linked to chromosome 13q12-13 in 1994 [8] and cloned 1 year later [9]. The *BRCA2* gene spans approximately 84 kb and is composed of 27 exons, which encodes a protein of 3418 amino acids. It contains eight BRC domains, which are involved in protein binding, including binding with Rad51 [10, 11]. BRCA1 and BRCA2 are both involved in the maintenance of genome stability [12, 13] and DNA repair via homologous recombination [14, 15],

but they are also suggested to play a role in transcriptional regulation [16], cell cycle control [17], chromatin remodelling [18] and ubiquitinylation [19].

The majority of *BRCA1* and *BRCA2* mutations are nonsense and small deletions/insertion mutations that lead to premature translation termination and production of truncated BRCA1 and BRCA2 proteins. Large genomic rearrangements were not reported until 1997 [20], but it has subsequently been reported that large genomic rearrangements—in particular in *BRCA1*—may account for 3–15% of all *BRCA1* and *BRCA2* mutations.

Using the multiplex ligation-dependent probe amplification (MLPA) assay, we report the identification of 15 breast and/or ovarian cancer patients from East Denmark with seven different large genomic rearrangements of which one is a specific Danish *BRCA1* founder mutation, two are novel deletions in *BRCA1*, and other two contain novel breakpoints.

Materials and methods

Patients

Following referral to oncogenetic counselling patients from Danish families with clustering of breast and/or ovarian cancer have since 1999 been offered *BRCA1* and *BRCA2* mutation screening according to the guidelines of the Danish Breast Cancer Cooperative Group (DBCG) (www.dbcg.dk). Families were classified as high-risk families based on manually evaluation of pedigrees and by use of tables and typically contained patients with breast cancer before the age of 40 years, with both breast and ovarian cancer, with two first degree relatives with breast cancer before the age of 50 years or ovarian cancer, with three first degree relative over two generation of which one was younger than 50 years, or patients with male breast cancer. Affected individuals or obligate carriers with a family history consistent with autosomal dominant inheritance of breast and/or ovarian cancer were screened for *BRCA1* and *BRCA2* mutations. Eligible for the current study were patients who received counselling between 1999 and 2007, and in whom a deleterious mutation in *BRCA1* and *BRCA2* not could be demonstrated by the initial pre-screening with denaturing high performance liquid chromatography (dHPLC) and sequencing. All together 642 probands were analysed for large genomic rearrangements by MLPA analysis. Blood samples were obtained from breast and ovarian cancer patients after informed consent and the original consent encompassed additional screening in case of new methods or targets. The family history was verified using the registry of the DBCG, hospital medical records and pathology reports and genetic counselling was provided for every family member.

MLPA analysis

Genomic DNA was purified from whole blood samples using the QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. MLPA analysis was performed according the manufactures instructions (MRC-Holland) using the SALSA P002 *BRCA1* and the SALSA P045 *BRCA2* MLPA kits. GeneMarker software (SoftGenetics) was used for fragment analysis. When a positive result (30% change) appeared the analysis was repeated (for *BRCA1* with MLPA kit P087). Moreover single exon deletions identified by MLPA analysis were sequenced using an ABI 3730 DNA analyzer (Applied Biosystems) using the BigDye terminator v1.1 cycler sequencing kit (Applied Biosystems) to exclude the presence of variants affecting the hybridization of the MLPA probes.

DNA breakpoint analysis

When possible deletions were verified by long range PCR using the Expand long template PCR kit (Roche) as recommended by the manufacturer. For the *BRCA1* exon 13 duplication, the genomic DNA was amplified with the following primers 5′-GATTATTTCCCCCCAGGCTA-3′ and 5′-AGATCATTAGCAAGGACCTGTG-3′ as described [21]. The samples were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. For the *BRCA1* exon 3–16 deletion, the genomic DNA was amplified with the following primers 5′-GAAAAAGTAA GAGACACCTATAG-3′ and 5′-CTTTATAAGCTGAGAG GTAACTAG-3′, for the *BRCA1* exon 5–7 deletion we used the following primers 5′-ACCTAGTCACCCCCTTCACC-3′ and 5′-CCAGCCCCAGAATGATTACTT-3′, for the *BRCA1* exon 17–18 deletion we used the primers 5′-GAATG TCCATGGTGGTGTCTGGC-3′ and 5′-GCCTGCATAAT TCTTGATGATCC-3′ and finally for the *BRCA2* exon 20 deletion the following primers 5′-GCTAACAGTACTCGG CCTGCTCGC-3′ and 5′-GCTTCTTTCCTTTAGAAATTA CCCAG-3′ were used.

In all these cases the samples were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Finally, the bands were purified and sequenced using an ABI3730 DNA analyzer (Applied Biosystems) using the BigDye terminator v1.1 cycler sequencing kit (Applied Biosystems). For genomic breakpoint determination the genomic reference sequences NC_000017 and NC_000013.9, and the coding DNA reference sequences NM_007294 and NM_000059 were used.

SNP chip analysis

Genomic DNA was applied to 250 K *Sty*I (~238.000 SNPs) SNP-microarray chips and processed according to the

manufacturer's instructions (Affymetrix). Briefly, 250 ng of genomic DNA was digested with *Sty*I and ligated to adapters. Adapter ligated DNA was amplified, purified, fragmented and labeled with biotin and hybridized to the arrays for 18 h. The Affymetrix 450 fluidics station and the Affymetrix 3000 G7 gene scanner were used to wash, stain and scan the arrays. The CEL files were analysed using the BRLMM algorithm from Affymetrix Genotyping analysis software (GTYPE). For the 9 individuals with the *BRCA1* exon 3–16 deletion we removed all SNPs with missing genotypes or with missing position annotation leaving 222.543 SNPs. Using the HapMap Ceph parents as a reference sample we estimated the co-ancestry between individuals using a moment approach [22]. The co-ancestry coefficient was lower than 5% between all pairs of individuals meaning that none of the individuals are closely related. We estimated the length of the possible shared haplotypes from the genotypes. We defined the possible shared haplotype as a region where all the genotypes are consistent with a single haplotype in the sense that there are no pairs of individuals that are homozygous for different alleles.

Results

To determine the frequency of large genomic rearrangements (LGRs) in *BRCA1* and *BRCA2* in East Denmark, we examined 642 breast and/or ovarian cancer patients previously screened negative for small disease causing mutations in *BRCA1* and *BRCA2* by MLPA analysis as recently described [23]. The MLPA analysis revealed 15 LGRs in *BRCA1* and *BRCA2,* including a *BRCA1* exon 3–16 deletion in nine patients with breast and/or ovarian cancer or family members with breast and/or ovarian cancer, a *BRCA1* exon 5–7 deletion in a patient with breast and ovarian cancer, a *BRCA1* exon 13 duplication in a patient with breast cancer, a *BRCA1* exon 17–18 deletion in a breast cancer patient with Iranian ancestors, a *BRCA1* exon 17–19 deletion in a patient with breast cancer, a *BRCA1* exon 19 deletion in a patient with breast and ovarian cancer, and a *BRCA2* exon 20 deletion in a male with pancreatic and breast cancer (Fig. 1a–g and Table 1). All *BRCA1* changes were verified in a new DNA sample using the P087 *BRCA1* MLPA control kit. Moreover, all single exon deletions were sequenced to exclude the presence of a nucleotide variant within the probe-binding site.

Since we previously identified deleterious mutations in *BRCA1* and *BRCA2* in 148 families, LGRs account for 9.2% (15/163) of all disease causing mutations identified in East Denmark. Of the 15 LGRs identified, 14 were in *BRCA1*, while only 1 was identified in *BRCA2*, showing that *BRCA1* LGRs account for 12.5% (14/112), while *BRCA2* accounts for 2% (1/51) out of all *BRCA1* and *BRCA2* disease causing mutations identified. The average age on onset for all female probands was approximately 36 years (excluding one woman without cancer and one with unknown age of breast cancer onset), while the average age on onset for the eight female patients with the *BRCA1* exon 3–16 deletion was approximately 35 years (Table 1). Most families also had family member with breast cancer and in 7 out of 14 families with *BRCA1* LGRs we find the occurrence of both breast and ovarian cancer (Table 1).

To determine the breakpoints for the deletions long-range PCR was performed. The size of the PCR products from the nine patients with the *BRCA1* exon 3–16 deletion was examined by agarose gel electrophoresis, and found to be identical (Fig. 2a). Sequencing showed that the genomic breakpoint was located at g.8655_55240del46586 (c.81-1018_4986+716del46586) (Fig. 2a). The *BRCA1* exon 5–7 deletion breakpoint was determined to be g.18296_23289 del4994 (c.136-623_441+1959del4994) (Fig. 2b), while the *BRCA1* exon 13 duplication was examined by PCR and yielded a band of approximately 1 kb showing the breakpoint to be identical to the recently published [21] (Fig. 2c). Moreover, the breakpoints for the *BRCA1* exon 17–18 deletion was determined to be located at g.55023_61749del6727 (c.4986+498_5152+171del6727) (Fig. 2d), whereas the breakpoints for the *BRCA1* 17–19 deletion and exon 19 was undetermined due to insufficient amounts of DNA. Finally, the breakpoint for the *BRCA2* exon 20 deletion was determined to be located at g.55520_56675del1156 (c.8531_8632+1054) (Fig. 2e).

To determine if the *BRCA1* exon 3–16 deletion represented a Danish founder mutation, we compared the SNP profiles of the affected families by analysis on a 500 K SNP-array. If the mutation was a founder mutation, the affected individuals are expected to share a single haplotype around the mutation. Figure 3 shows the lengths of possible shared haplotypes on chromosome 17. We note a 5 Mb long shared haplotype around the *BRCA1* consisting of 300 SNPs. This is by far the largest possible shared haplotype in the genome when removing possible shared haplotypes with less than 100 SNPs (see supplementary Fig. 1). Based on the possible shared haplotypes the nine individuals positive for the *BRCA1* mutation are more closely related in the *BRCA1* region, than any other region on the genome. We therefore infer that the mutation in the *BRCA1* region happened once and segregated in the population to these nine individuals. This can be seen even though none of the individuals showed any close relatedness, when considering the whole genome variation.

Discussion

The human genome contains about one Alu repeat every 5 kb and these may cause large genomic rearrangements

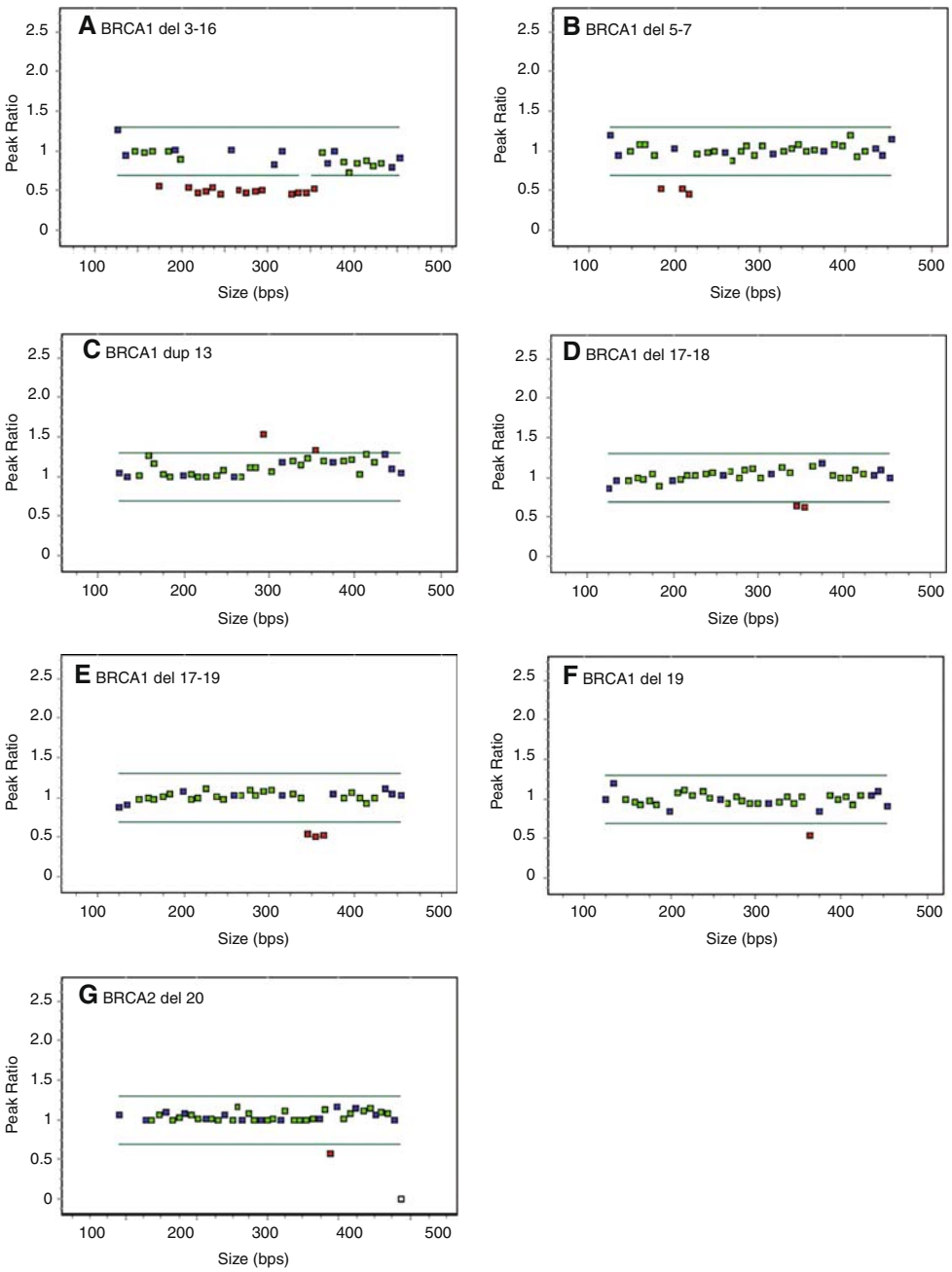

Fig. 1 Multiplex ligation-dependent probe amplification (MLPA) analysis of *BRCA1* and *BRCA2* large genomic rearrangements. MLPA analysis of the *BRCA1* exon 3–16 deletion (**a**), the *BRCA1* exon 5–7 deletion (**b**), the *BRCA1* exon 13 amplification (**c**), the *BRCA1* exon 17–18 deletion (**d**), the *BRCA1* exon 17–19 deletion (**e**), the *BRCA1* exon 19 (**f**), and the *BRCA2* exon 20 deletion (**b**) are shown. The green squares are *BRCA1* and *BRCA2* probes, the blue squares are control probes, while the deleted or duplicated exons are indicated by red squares

and homologous recombination events. The *BRCA1* gene spans ~81 kb DNA and almost 42% of the genomic sequence is composed of Alu sequences. *BRCA2* is slightly larger but contains fewer Alu repeats and it is presumed, that this is the reason why LGRs are less frequently observed in *BRCA2*. In our consecutive series of Danish high risk breast and ovarian cancer families, LGRs overall accounts for 9.2% of all disease-causing mutations in *BRCA1* and *BRCA2* taken as a group and for 12.5% of all *BRCA1* disease causing mutations. This is higher than previously reported from West Denmark, where LGRs were identified in 2.3% of all disease causing *BRCA1* and *BRCA2* mutations, and 3.8% of *BRCA1* disease-causing mutations [24], and from Finland [25] and the French part of Canada [26] where no LGRs were found. In contrast the data are more in agreement with results from France (9.5%) [27], Spain (8.2%) [28], Germany (8%) [29] and Czech Republic (6%) [30], but lower than observed in the Netherlands (27–36%) [23, 31] and Italy (19%) [32]. Only one family with a LGR in *BRCA2* was identified, in accordance with studies from other populations, which found no families with *BRCA2* LGRs [25, 33, 34]. The mutation was identified in a male patient, in agreement with the finding that LGRs in *BRCA2* are more frequent in

Table 1 *BRCA1* and *BRCA2* large genomic arrangements in families from East Denmark with hereditary breast and/or ovarian cancer

Family ID	Gene	MLPA	Sex of proband	Phenotype and age at diagnosis	Family history of breast and ovarian cancer and age at diagnosis	Other cancers in the families	Genomic and cDNA alteration
01836	*BRCA1*	Del exon 17–18	Female	BC 33	M BilatBC <50, S BC 28	Unknown	**g.55023_61749del6727** **c.4986+498_5152+171del6727**
14242	*BRCA1*	Del exon 3–16	Female	BC 31	M BC 44+49, MA BC 42	Lung cancer	g.8655_55240del46586 c.81-1018_4986+716del46586
15371	*BRCA1*	Del exon 3–16	Female	OC 41	PA OC 39, PC OC 38	None	g.8655_55240del46586 c.81-1018_4986+716del46586
20164	*BRCA1*	Del exon 3–16	Male		M BC >50 D BC 40+43	None	g.8655_55240del46586 c.81-1018_4986+716del46586
32682	*BRCA1*	Del exon 3–16	Female	BC 40, OC 59		None	g.8655_55240del46586 c.81-1018_4986+716del46586
35746	*BRCA1*	Del exon 3–16	Female	BC 32	PGM BC 33 PA BC 40, OC 53	None	g.8655_55240del46586 c.81-1018_4986+716del46586
55202	*BRCA1*	Del exon 3–16	Female	BC 28, OC 47		None	g.8655_55240del46586 c.81-1018_4986+716del46586
58701	*BRCA1*	Del exon 3–16	Female	BC 33	M Gyn	None	g.8655_55240del46586 c.81-1018_4986+716del46586
59243	*BRCA2*	Del exon 20	Male	PC 65, BC 86,	M BC 85, S BC 55, S BC 70	Lung cancer, sarcoma	**g.55520_56675del1156** **c.8531_8632+1054**
60935	*BRCA1*	Amp exon 13	Female	BC 39	M BC 34	None	ND
61096	*BRCA1*	Del exon 3–16	Female	BC 40	S BC 38	Colon cancer	g.8655_55240del46586 c.81-1018_4986+716del46586
67381	*BRCA1*	Del exon 17–19	Female		M BC 51, OC 56, MA OC 65, MA BC 52, MA OC 56, BC 72	Ventricle cancer	ND
71406	*BRCA1*	Del exon 5–7	Female	BC 49, OC 53	S OC 44, MA OC 37	None	**g.18296_23289del4994** **c.136-623_441+1959del4994**
71639	*BRCA1*	Del exon 3–16	Female	BilatBC 34, 37	M BC 35 MGM Gyn	Unknown	g.8655_55240del46586 c.81-1018_4986+716del46586
83947	*BRCA1*	Del exon 19	Female	BC, OC	D BC, D BC	Unknown	ND

Gyn, undefined gynecological cancer; BC, breast cancer; OC, ovarian cancer; PC, prostate cancer; MGM, Maternal grand mother; PGM, paternal grandmother; M, mother; S, sister; D, daughter; PA, paternal aunt; PC, paternal cousin; MA, maternal aunt; MC, maternal cousin. For genomic breakpoint determination the genomic reference sequence NC_000017 and NC_000013.9, and the coding DNA reference sequence NM_007294 and NM_000059 were used. The novel mutations are indicated in bold. ND; Not determined

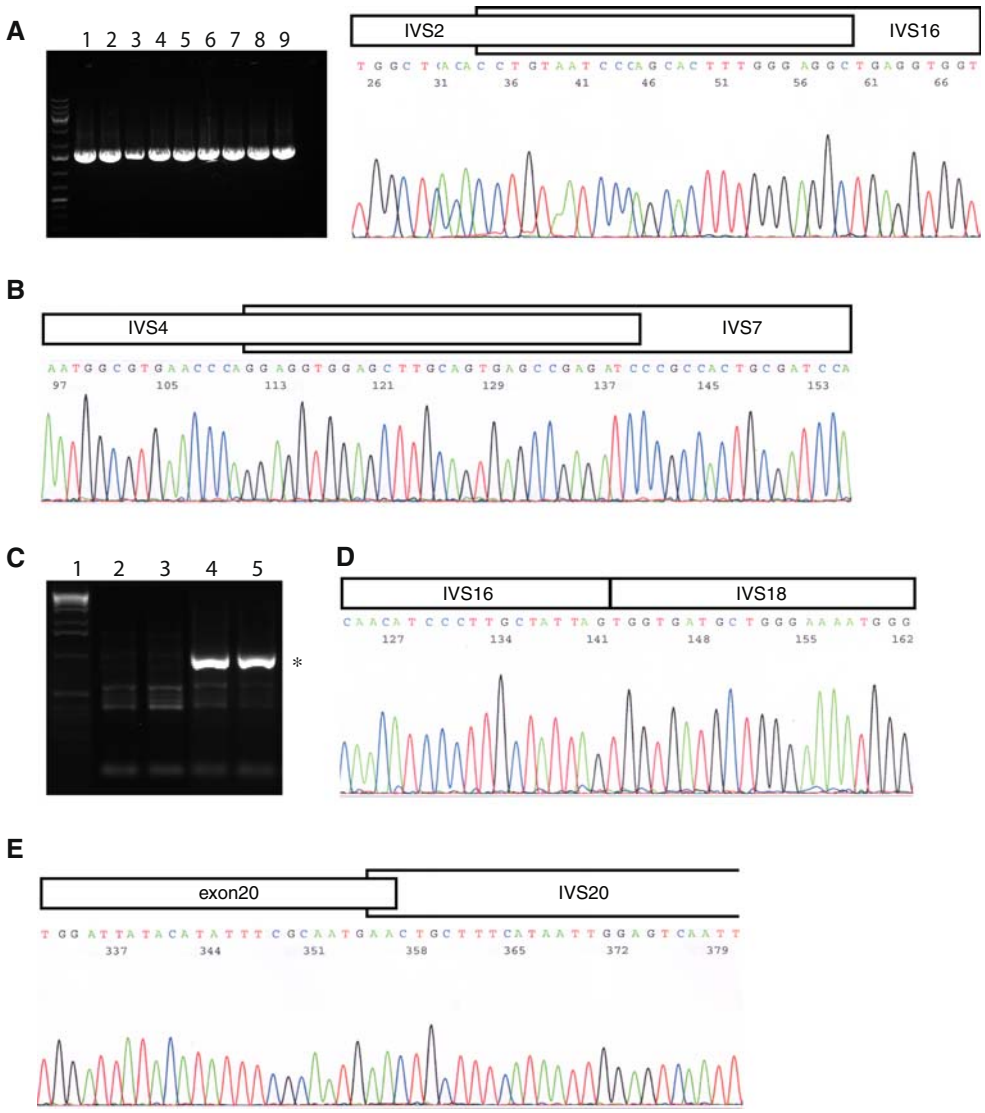

Fig. 2 Breakpoint analysis of *BRCA1* and *BRCA2* large genomic rearrangements. (**a**) Long-range PCR analysis and gel electrophoresis was performed on the 9 patients harbouring the *BRCA1* exon 3–16 deletion. The resulting PCR fragments are shown to the left. The breakpoint was determined by sequencing (on the right) and designated g.8655_55240del46586 (c.81-1018_4986+716del46586) (**b**) The breakpoint for the *BRCA1* exon 5–7 deletion was determined by long-range PCR and sequencing to be g.18296_23289del4994 (c.136-623_441+1959del4994). (**c**) The *BRCA1* exon 13 duplication was examined by PCR analysis and gel electrophoresis and yielded a band of approximately 1 kb showing that the breakpoint is identical to the recently published [21]. The size marker is shown in lane 1, control DNA in lane 2 and 3, and patient DNA in lane 4 and 5. (**d**) The breakpoint for the *BRCA1* exon 17–18 deletion was determined by long-range PCR and sequencing to be g.55023_61749del6727 (c.4986+498_5152+171del6727). (**e**) Finally, the breakpoint for the *BRCA2* exon 20 deletion was determined by long-range PCR and sequencing to be g.55520_56675del1156 (c.8531_8632+1054). The site of crossover is indicated in all cases

families with male breast cancer [35, 36]. The clinical feature of the families exhibiting LGRs was not different from those exhibiting other types of mutations. As previously reported many of the families with mutation in *BRCA1* contain both breast and ovarian cancer and a young age at diagnosis [37]. We find the occurrence of both breast and ovarian cancer in 7 out of 14 families with *BRCA1* LGRs and that the average age of cancer onset for female probands was approximately 36 years.

Of the seven different LGRs found in East Denmark, five have previously been described. In particular the *BRCA1* 3–16 deletion has previously been reported in two patients from West Denmark [24] and in a ovarian cancer patient from Britain/USA [38]. The 9 probands exhibited the same breakpoint (g.8655_55240del46586/c.81-1018_4986+716del46586), that is identical to that reported by others [24]. The nine families share a 5 Mb fragment containing the mutation, which suggests that it represents a

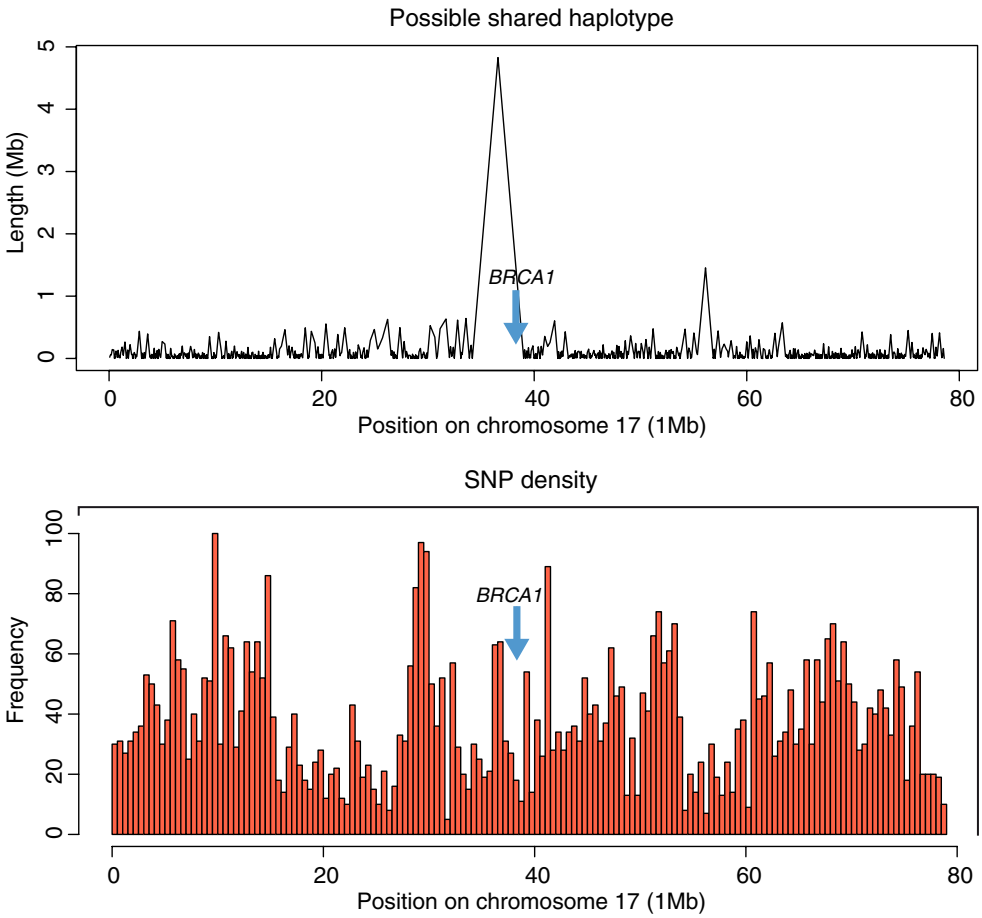

Fig. 3 SNP analysis of families harbouring the *BRCA1* exon 3–16 deletion. The lengths of the possible shared haplotypes are shown as lines in the top figure and the *BRCA1* gene is shown in green at position 38,449,840-38,530,994. The bottom picture shows the SNP density on chromosome 17

founder mutation and not a mutation hot-spot. LGR founder mutations have also been reported in other populations, including *BRCA1* exon 13 and exon 22 deletions in the Netherlands [31]. The *BRCA1* exon 5–7 deletion was found in a patient with British ancestors. This deletion has previously been identified in both a German [39], and an Italian [32] family with breast and ovarian cancer. The deletion introduces a frameshift, which is predicted to result in a truncation at codon 163 in BRCA1. The breakpoint in our patient was identified to be g.18296_23289del4994 (c.136-623_441+1959del4994), which is different to those previously identified [39], indicating that the involved intronic regions are inclined to rearrangements. The *BRCA1* exon 13 duplication has previously been identified in Britain, Sweden, Germany and Asia [21, 39–41]. It is known to include a 6-kb fragment containing two exon 13 fragments [21], introducing a frameshift. We were able to verify the exon 13 duplication by PCR as described [21], implying that the breakpoint is identical to the previously reported duplication. The *BRCA1* exon 17–19 deletion has previously been described in an American/European breast and ovarian cancer family [42], but no breakpoint was identified. This deletion does not create a frameshift, but deletes amino acids 1663–1731 which contains one of the BRCT domains involved in protein interactions with BRIP1, CtIP, and Abraxas [5–7]. Interestingly, this region has also been reported to be triplicated [23]. Finally, we identified a single LGR in *BRCA2*. This was a *BRCA2* exon 20 deletion in a male with pancreas and breast cancer. Three female family members moreover had late onset breast cancer. Long-range PCR analysis revealed that the patient had a 1.156 bp genomic deletion. A *BRCA2* exon 20 deletion has previously been observed in Italy in a family with breast cancer, including male breast cancer [43]. In that study the breakpoint was defined as a 4.953 bp deletion (g.56447_61399del4953), which is different to the 1156 bp deletion identified in our study (g.55520_56675del1156/c.8531_8632+1054). Finally the *BRCA1* exon 17–18 and the *BRCA1* exon 19 deletions have not been described before. The breakpoint in the *BRCA1* exon 17–18 deletion occurred at g.55023_61749del6727 (c.4986+498_5152+171del6727). Both deletions introduce a frameshift and introduce stop codons at codon 1719 and at codon 1732 in BRCA1, respectively.

In conclusion, we show that LGRs in *BRCA1* and *BRCA2* are common in East Denmark and account for 9.2% of the disease causing mutations. We also report two novel deletions and show that the *BRCA1* exon 3–16 deletion is a

founder mutation. Finally our study suggests that, even though the number of LGRs in *BRCA2* in East Denmark is low, MLPA analysis of *BRCA2* should be included in the screening of high-risk breast and/or ovarian cancer families.

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