Large Genomic Rearrangements of \textit{BRCA1} and \textit{BRCA2} among Patients Referred for Genetic Analysis in Galicia (NW Spain): Delimitation and Mechanism of Three Novel \textit{BRCA1} Rearrangements

Laura Fachal\textsuperscript{1}, Ana Blanco\textsuperscript{1}, Marta Santamariña\textsuperscript{2}, Angel Carracedo\textsuperscript{1}, Ana Vega\textsuperscript{1,*}

\textsuperscript{1} Fundación Pública Galega de Medicina Xenómica-SERGAS. Grupo de Medicina Xenómica, CIBERER, IDIS, Santiago de Compostela, Spain, \textsuperscript{2} Grupo de Medicina Xenómica-USC, University of Santiago de Compostela, CIBERER, IDIS, Santiago de Compostela, Spain

Abstract

In the Iberian Peninsula, which includes Spain and Portugal, large genomic rearrangements (LGRs) of \textit{BRCA1} and \textit{BRCA2} have respectively been found in up to 2.33\% and 8.4\% of families with hereditary breast and/or ovarian cancer (HBOC) that lack point mutations and small indels. In Galicia (Northwest Spain), the spectrum and frequency of \textit{BRCA1}/\textit{BRCA2} point mutations differs from the rest of the Iberian populations. However, to date there are no Galician frequency reports of \textit{BRCA1}/\textit{BRCA2} LGRs. Here we used multiplex ligation-dependent probe amplification (MLPA) to screen 651 Galician index cases (out of the 830 individuals referred for genetic analysis) without point mutations or small indels. We identified three different \textit{BRCA1} LGRs in four families. Two of them have been previously classified as pathogenic LGRs: the complete deletion of \textit{BRCA1} (identified in two unrelated families) and the deletion of exons 1 to 13. We also identified the duplication of exons 1 and 2 that is a LGR with unknown pathogenicity. Determination of the breakpoints of the \textit{BRCA1} LGRs using CNV/SNP arrays and sequencing identified them as NG_005905.2:g.70536_180359del, NG_005905.2:g.90012_97270dup, and NC_000017.10:g.41230935_41399840delinsAluSx1, respectively; previous observations of \textit{BRCA1} exon1-24del, exon1-2dup, and exon1-13del LGRs have not characterized them in such detail. All the \textit{BRCA1} LGRs arose from unequal homologous recombination events involving Alu elements. We also detected, by sequencing, one \textit{BRCA2} LGR, the Portuguese founder mutation c.156_157insAluYa5. The low frequency of \textit{BRCA1} LGRs within \textit{BRCA1} mutation carriers in Galicia (2.34\%, 95\% CI: 0.61–7.22) seems to differ from the Spanish population (9.93\%, 95\% CI: 6.76–14.27, \textit{P}-value = 0.013) and from the rest of the Iberian population (9.76\%, 95\% CI: 6.69–13.94, \textit{P}-value = 0.014).

Citation: Fachal L, Blanco A, Santamariña M, Carracedo A, Vega A (2014) Large Genomic Rearrangements of \textit{BRCA1} and \textit{BRCA2} among Patients Referred for Genetic Analysis in Galicia (NW Spain): Delimitation and Mechanism of Three Novel \textit{BRCA1} Rearrangements. PLoS ONE 9(3): e93306. doi:10.1371/journal.pone.0093306

Editor: Amanda Ewart Toland, Ohio State University Medical Center, United States of America

Received December 30, 2013; Accepted February 28, 2014; Published March 31, 2014

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Funding: This work was supported by grants to AV from the Xunta de Galicia (10 PXXB 9101 297 PR) and the Fundación Mutua Madrileña. LF is supported by the Isabel Barreto programme of the Xunta de Galicia and the European Social Fund. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: ana.vega@usc.es

Introduction

The two major high-penetration breast cancer susceptibility genes, \textit{BRCA1} [1] and \textit{BRCA2} [2], account for approximately 26\% of all cases of hereditary breast and/or ovarian cancer (HBOC) [3]. To date, some 1,700 \textit{BRCA1} variants and 1,900 \textit{BRCA2} variants have been reported (Breast Cancer Information Core database, http://research.nhgri.nih.gov/bic/). However, only 81 \textit{BRCA1} variants and 17 \textit{BRCA2} variants are large genomic rearrangements (LGRs) [4], and the prevalence of \textit{BRCA1}/\textit{BRCA2} LGRs varies widely among different populations, mainly due to the existence of founder rearrangements. For example, in the Netherlands, \textit{BRCA1} LGRs constitute up to 27\% of all \textit{BRCA1} mutations (see Sluiter et al. [4] and references therein), whereas to date, only one \textit{BRCA2} LGR has been reported in a proband of Dutch and German ancestry [5]. On the contrary, in Portugal \textit{BRCA1} LGRs represents the ~6\% of \textit{BRCA1} mutations while, due to the Portuguese \textit{BRCA2} founder mutation c.156_157insAluYa5, the frequency of \textit{BRCA2} LGRs is the highest reported to date (57.89\% of \textit{BRCA2} mutations) [6–8].

Of the published studies of the frequency of \textit{BRCA1}/2 LGRs in the Iberian Peninsula or regions thereof [6–17], none has specifically examined the population of Galicia (NW Spain), a region with a distinct genetic identity attributable to its historical relative isolation, its cultural identity, and the occurrence of a marked population bottleneck around 1000 years ago [18]. This population features a number of founder mutations [18–20], including a \textit{BRCA1} mutation, c.211A>G (referred to NM_007294.3; BIC 330A>G), which is present in more than 50\% of Galician HBOC families with \textit{BRCA1}/2 mutations [21].

In view of to date there are no Galician frequency reports of \textit{BRCA1}/\textit{BRCA2} LGRs and the observed differences between Galicia and the rest of Spain in regard to the spectrum and prevalence of point mutations of \textit{BRCA1} and \textit{BRCA2}, we decided to investigate whether a similar situation holds for \textit{BRCA1}/2 LGRs. We accordingly screened for \textit{BRCA1}/2 LGRs among
Galician families referred for genetic examination of BRCA1/2. Here we describe three novel BRCA1 LGRs, propose likely originating mechanisms, and compare the frequency of LGRs in Galicia with published results for the remainder of the Iberian Peninsula.

Materials and Methods

Participants
Our reference laboratory handles essentially all Galician patients referred for evaluation of the possibility of HBOC by means of DNA analysis. Between 1997 and 2012 we examined BRCA1/2 in 830 patients referred to us in accordance with the criteria established in Galician oncological guidelines [22], which currently recommend referral if patients have (i) three or more first degree relatives who have suffered breast or ovarian cancer, (ii) two affected first degree relatives if one was aged <40 years at diagnosis, (iii) two affected first or second degree relatives if both suffered breast cancer at age <50 years, or if one suffered bilateral breast cancer and one was aged <50 years at diagnosis, or if at least one of these cancers was ovarian or a male breast cancer, (iv) age <30 years at diagnosis of a breast cancer, (v) both breast and ovarian cancer, (vi) bilateral breast cancer diagnosed before the age of 40 years, or (vii) a family history of deleterious mutation of a breast cancer susceptibility gene. These patients were screened for the founder mutations BRCA2 c.156_157insAluYa5 and BRCA1 c.211A>G (BIC 330A>G) using protocols respectively described by Peixoto et al. [7] and Vega et al. [21], after which other BRCA1/2 mutations were sought by bi-directional sequencing of exons and flanking intronic splice sites. Of the 830 referred patients, 125 were found to have point mutations or small indels in BRCA1, and 54 point mutations or small indels in BRCA2. The 651 with no point mutations or small indels were included in the present study, as were members of their families when this was appropriate and possible.

Relationships between index cases were investigated through the genealogical tree, which include at least three generations, and the family name that in Spain included the surname from the father and also from the mother.

Ethics Statement
The study conformed to Spanish biomedical research legislation (Ley 14/2007) and was approved by the Galician Ethical Committee for Clinical Research. All participants gave written informed consent.

LGR screening
Screening for LGRs in BRCA1 and BRCA2 was performed by multiplex ligation-dependent probe amplification (MLPA). The commercial BRCA1 kits P002 (primary screening) and P087 (confirmatory) and the BRCA2 kit P045 were used in accordance with the manufacturer’s instructions [23]. Fragment electrophoresis was performed on an Applied Biosystems 3730 xl DNA analyzer using GeneScan 500 LIZ size standards (Applied Biosystems, USA) and at least 24 samples in each run, and the resulting data were analyzed using GeneMapper software (Applied Biosystems, USA). Visual peak pattern evaluation was carried out following the manufacturer’s recommendations. After exclusion of samples failing the first quality control, the remaining samples were analyzed using Cofalysyer v8 software in “direct analysis” normalization mode using concurrently run samples as the reference set and taking the medians of the corresponding normalized probe signal ratios. When all sample signals had been normalized in this way, any samples with aberrant probe signals (≥0.15 standard deviations from the mean) were removed, the whole normalization process was repeated, and so on until the standard deviations of all probe signals were <0.15.

SNP arrays and analysis of breakpoint regions
LGRs were characterized using the Cytogenetics Whole-Genome 2.7 M Array in combination with the Genome-Wide Human SNP Array 6.0, or alternatively the CytoScan HD Array (all from Affymetrix). The results were analyzed with the Chromosome Analysis Suite (Affymetrix), the breakpoint regions delimited by the array markers were examined in the UCSC Genome Browser (http://genome.ucsc.edu/; assembly NCBI37/hg19), and repetitive sequences in these regions were identified using RepeatMasker [24] within the Genome Browser. Breaks were pinpointed by sequencing as next described.

Sequencing
PCR primers were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/); primer sequences and PCR conditions are described in the Table S1. Sequencing was performed using BigDye Terminator v3.1 sequencing kits (Applied Biosystems, USA). Electrophoresis was carried out on an ABI 3730 xl DNA analyzer (Applied Biosystems, USA).

Statistical analyses
Association tests were performed using two-degrees of freedom Pearson’s chi-square test with Yates correction. Statistical analyses were performed using the statistical package stats with the software R v3.0.2. A nominal P-value of 0.05 was considered significant.

Results
Among the 651 apparently unrelated index cases studied we found four different LGRs, three in BRCA1 by MLPA and one in BRCA2 by sequencing (Table 1).

Exon1-2dup
A duplication of BRCA1 exons 1-2 was identified in a 66-year-old woman in whom breast cancer was diagnosed at age 64 years. Previously, her three sisters had developed breast cancer (at ages 35, 49 and 59 years), as had her two maternal aunts (according to the family, though this was not confirmed) (Family I, Fig. 1). The SNP array bracketed the downstream breakpoint but not the upstream one (Fig. 1c; throughout this paper, “upstream” and “downstream” respectively refer to the directions of decreasing and increasing genomic coordinates). However, sequencing identified a hybrid Alu element in which AluYk4 and AluY2, two of the ten Alu elements identified by RepeatMasker in the bracketed region and its upstream vicinity (Fig. 1d), overlapped by 48 nt (Fig. 1c), indicating the tandem repetition of BRCA1 exons 1A, 1B and 2 through duplication of the 7,259-nt sequence NG_005905.2:g:90012_97270. The origin of the duplication was thus an unequal homologous recombination event that created the hybrid Alu element at the point of recombination. Note that since non-coding BRCA1 exon 1A shares part of its sequence with non-coding NBR2 exon 1 (the two exons together forming a bidirectional promoter regulated by different transcriptional repressor factors), the duplication of BRCA1 exons 1 and 2 also means the duplication of part of the neighboring gene (NBR2 exon 1). Unfortunately, a co-segregation study could not be performed since the affected family members were deceased.

Exon1-24del
The complete deletion of BRCA1 was identified in two presumably unrelated Galician families. In Family II the index
Table 1. Large genomic rearrangements in the *BRCA1* gene identified in the Galician population.

| Family | Gene | BIC LGR designation | Clinical Significance | Detection method | Confirmation method | Other affected genes | HGVS designation* | Size (bp) | Previously reported LGR affecting the same exons | Size (bp) | Geographical region |
|--------|------|---------------------|-----------------------|------------------|---------------------|---------------------|-------------------|-----------|-----------------------------------------------|-----------|---------------------|
| I      | *BRCA1* | exon1-2dup | VUS | MLPA | CNV/SNP array, sequencing | NRB2 | NG_005905.2: g.90012_97270dup | 7,259 | Del Valle et al. [12] | nd | Spain |
| II, III | *BRCA1* | exon1-24del | Deleterious | MLPA | CNV/SNP array, sequencing | NR2 | NG_005905.2: g.70536_180359del | 109,824 | De la Hoya [9] | nd | Spain |
|        |       |                     |                      |                  |                     |                    |                   |           | Blay et al. [16] | nd | Asturias (Northern Spain) |
| IV     | *BRCA1* | exon1-13del | Deleterious | MLPA | CNV/SNP array, sequencing | NR2, NBR1, TME106A | NC_000017.10: g.41230935_41399840delinsAluSx1 | 168,905 | Del Valle et al. [12] | ~250,000 | Spain |
|        |       |                     |                      |                  |                     |                    |                   |           | Blay et al. [16] | nd | Asturias (Northern Spain) |
| V      | *BRCA2* | 384insAlu | Deleterious | Sequencing | Primer-specific sequencing | - | NG_012772.1: g.8686_8687insAluYaS | ~350 | Peixoto et al. [8] | ~350 | Portugal |

VUS: variant of uncertain significance; nd: non described.

*According to reference sequences NG_005905.2 or NG_012772.1, except for *BRCA1* exon1-13del, since it does not extend far enough upstream to describe the deletion of exons 1-13.

doi:10.1371/journal.pone.0093306.t001
patient was a 49-year-old woman in whom breast cancer was diagnosed at age 45 years (Fig. 2a). Breast cancer had also been diagnosed in her paternal grandmother (at age 60 years), in a half-cousin on her father’s side (at age 30 years), and in her great-grandmother, although this last case was not confirmed. In Family III the deletion of exons 1-24 was detected in a 50-year-old woman who had sought genetic evaluation following identification of this deletion in her sister, in whom breast cancer had been diagnosed at age 46 years (the sister had been evaluated in another laboratory and pedigree data were not made available to us). In the present study, SNP array analysis showed that in both the affected families the deletion includes **NBR2** and **BRCA1** (Fig. 2c), and in both cases amplification and sequencing of the junction region identified a segment in which AluSq2 and AluY, two of the five Alu elements located by RepeatMasker in the breakpoint regions (Fig. 2d), shared a sequence of 20 nucleotides (Fig. 2e). We accordingly identify this LGR as the 109,824 bp deletion NG_005905.2:g.70536_180359del, and as attributable to unequal homologous recombination.

**Exon1-13del**

Deletion of **BRCA1** exons 1-13 was detected in a 49-year-old woman in whom bilateral breast cancer was diagnosed at 35 and 40 years of age. One of her paternal cousins and a maternal aunt had also developed breast cancer (at ages 35 and 40 years, respectively), and another paternal cousin developed ovarian cancer at age 40 years (Fig. 3a). In the downstream breakpoint region delimited by the SNP array (Fig. 3c) RepeatMasker showed two Alu elements (Fig. 3d). In the putative upstream breakpoint region it found no repetitive elements, but did find several further upstream. Using a forward primer hybridizing on a non-repetitive sequence (Table S1), we were able to identify a segment in which one of the downstream Alu elements, AluSc8, shares 41 nucleotides with an AluSx1 (Fig. 3e), the initial 173-nt segment

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Figure 1. **BRCA1 exon1-2dup.** a) Pedigree of Family I. +: mutation carrier; -: mutation non-carrier. b) MLPA normalized ratio results. Dark blue: reference signal for each probe created as described under Material and Methods. Light blue: sample probes with ratios ≥1.3 or ≤-0.7. c) Location of the downstream breakpoint region on the forward strand, as delimited (arrows) by SNP array results. Genes on the forward strand are shown in blue and genes on the reverse strand in green. d) Repetitive elements identified by RepeatMasker in the breakpoint region. e) Overlapping AluY and AluYk4 sequences at the junction between the repeated sequences containing exons 1 and 2.

doi:10.1371/journal.pone.0093306.g001
of which is homologous with the initial segment of AluSq2, the first upstream Alu element (see Figure S1). This LGR therefore seems to have arisen through an unequal homologous recombination event involving the deletion of the 168,905-bp sequence NC_000017.10:41230935_41399840 and its replacement with AluSx1 (doubtless favored by the homology with AluSq2). 384insAlu

Routine screening detected the Portuguese founder mutation BRCA2 c.156_157insAluYa5 in a Galician woman in whom breast cancer was diagnosed at 41 years of age. Her niece, her deceased sister, her father and two aunts (one on each side) had also developed breast cancer (Fig. 4).

In the various studies describing BRCA1 and/or BRCA2 LGRs in diverse regions of the Iberian Peninsula (Table 2), the reported frequency of BRCA1 LGRs among HBOC families without point mutations or small indels ranges from 0.48% to 2.33%, and that of BRCA2 LGRs from 0% to 8.4%. The difference between the deleterious LGRs frequency in Galicia and in Spanish population or in the rest Iberian populations is statistically significant (Table 3. Chi-square test with Yates correction P-value = 0.013 or 0.014, respectively).

Discussion

In the present study we have identified three LRGs in BRCA1 and one in BRCA2. The duplication of exons 1 and 2, that is a variant of unknown significance, has been reported previously in a Spanish HBOC family, but was not characterized in detail [12]. Therefore, although it is plausible that both rearrangements share the same breakpoint, it cannot be assessed. The complete deletion of BRCA1 has in the past been reported five times, three cases concerning Spaniards [9,16,25], one a Central European patient [26] and the fifth a German [27], but only in one of these cases,
Figure 3. BRCA1 exon1-13del. a) Pedigree of Family IV. +: mutation carrier; -: mutation non-carrier. b) MLPA normalized ratio results (color key as for Fig. 1). c) Location of breakpoint regions on the forward strand, as delimited (arrows) by SNP array results (gene color key as for Fig. 1). d) Repetitive elements identified by RepeatMasker in the breakpoint regions, with a red frame highlighting the AluSq2 element replaced by AluSx1 in the patient. e) Overlapping AluSx1 and AluSc8 sequences at the junction (red letters indicate deleted segments, and forward and reverse sequences are shown because of the poly-A tail of AluSc8).

doi:10.1371/journal.pone.0093306.g003

Figure 4. BRCA2 c.156_157insAluYa5. Pedigree of Family V. +: mutation carrier; -: mutation non-carrier.

doi:10.1371/journal.pone.0093306.g004
| Study                      | NBRCA- | NBRCA+ | NBRCA2- | Geographical region     | NLGR | % (95% CI) | % (95% CI) | NBRCA- | NBRCA+ | NBRCA2- | NBRCA+ | NBRCA2- | NBRCA+ | NBRCA2- | NBRCA+ | NBRCA2- | NBRCA+ |
|---------------------------|------------------|--------|---------|------------------------|------|------------|------------|--------|--------|---------|--------|---------|--------|---------|--------|---------|--------|
| De la Hoya et al. 2006 [9] | 285              | 73     | ne      | Spain                  | 6    | 2.11 (0.86–4.75) | 8.22 (3.39–17.65) | ne     |        |         |        |         |        |         |        |         |        |
| Gutierrez-Enríquez et al. 2007 [10]  
* | 335              | ne     | na      | Spain                  | ne   | 5          | 1.49 (0.55–3.65) | na     |        |         |        |         |        |         |        |         |        |
| Miramar et al. 2008 [11] | 44               | 8      | 0       | Aragon (Northeast Spain)| 1   | 2.27 (0.12–13.51) | 12.50 (0.66–53.32) | 0      |        |         |        |         |        |         |        |         |        |
| Del Valle et al. 2010 [12]  
* | 257              | na     | na      | Spain                  | 6    | 2.33 (0.95–5.26) | na         | 2      | 0.78   | 0.14–3.09| na     |         |        |         |        |         |        |
| Rodriguez et al. 2010 [13] | 207              | na     | na      | Catalonia (Northeast Spain)| 1   | 0.48 (0.03–3.01) | na         | 1      | 0.48   | 0.03–3.08| na     |         |        |         |        |         |        |
| Peixoto et al. 2006 and 2011 [6,8]  
* | 79/131           | 15     | 19      | Portugal               | 1    | 1.27 (0.07–7.82) | 6.67 (0.35–33.97) | 11     | 8.4    | 4.47–14.87| 57.89  | 33.97–78.88|    |        |        |         |
| Ruiz de Garibay et al. 2012 [14] | 813          | ne     | na      | Spain                  | ne   | 7          | 0.86 (0.38–1.85) | na     |        |         |        |         |        |         |        |         |        |
| Juan Jiménez et al. 2013 [15] | 1471           | 155    | 155     | Valencian Community (Eastern Spain)| 17  | 1.16 (0.70–1.89) | 10.97 (6.71–17.24) | 1      | 0.07   | 0.00–0.44| 0.65   | (0.03–4.08) |    |        |        |         |
| Blay et al. 2013 [16] | 200              | 36     | 0       | Asturias (Northern Spain)| 3   | 1.50 (0.39–4.68) | 8.33 (2.18–23.59) | 0      |        |         |        |         |        |         |        |         |        |
| Present study             | 651              | 128    | 55      | Galicia (Northwest Spain)| 4   | 0.61 (0.20–1.68) | 2.34 (0.61–7.22) | 1      | 0.15   | 0.01–0.99| 1.81   | (0.10–11.18) |    |        |        |         |

NBRCA-: number of families without BRCA1/2 point mutations or small indels included in the study. NBRCA+: number of families with BRCA1 mutations; NBRCA2+: number of families with BRCA2 mutations; NLGR: number of families with LGRs; ne: non evaluated; na: non available.

*The number of families with point mutations or small indels was not stated for each gene.

Data for BRCA1 LGRs is extracted from Peixoto et al. 2006 [6], whereas data for BRCA2 LGRs is extracted from Peixoto et al. 2011 [8].

Excluding BRCA1 exon1-2dup, considered as of unknown pathogenicity. If this LGR is pathogenic, this frequency becomes 3.10%.

doi:10.1371/journal.pone.0093306.t002
that identified a de novo BRCA1 deletion, were the breakpoints characterized [25]. It is interesting that none of the three complete BRCA1 deletions with published length estimates can share both breakpoints, their lengths being <110 kb (this work), 259–345 kb [27], and ~150 kb [25] (this last deletion extending from the beginning of NBR1 to VAT1 and including the whole of RND2, ψBRCA1, BRCA1 and NBR2). The deletion of BRCA1 exons 1-13 has previously been reported in three families, two Spanish [12,16] and one Finnish [28]. It is unclear whether either of the breakpoints of this deletion (NC_000017.10:41230935_41399840) coincides with or lies close to the corresponding breakpoint of the approximately 250 kb exon1-13del LGR reported by del Valle et al. [12]: both these deletions eradicate NBR2, NBR1 and TMEM106A as well as BRCA1 exons 1-13. However, the present LGR does not affect ARL4D, whereas Del Valle et al. [12] only identified their downstream breakpoint as lying somewhere between exon 6 of TMEM106A and exon 2 of ARL4D. Concerning the BRCA2 c.156_157insAluYα5 mutation, Peixoto et al. [8] recently reported finding this mutation in only three out of 5,294 families living outside Portugal all three of which had emigrated relatively recently from Portugal. This is therefore, as far as we know, the first report of c.156_157insAluYα5 in a family not known to be of Portuguese origin. However, recent generations of our patient’s family have resided near the frontier between Spain and northern Portugal, where a high frequency of this founder mutation has been reported [7]. Although the absence of Portuguese ancestors in the past four generations has been reported by the family, the estimated age of the mutation, 561±215 years (estimated by the study of 19 SNPs and nine microsatellite markers spanning ~2 Mb within and around BRCA2 [8]) makes plausible a Portuguese origin for the mutation in our family.

Clinical classification of the identified LGRs

BRCA1 deletions of exons 1-13 and 1-24 are considered pathological LGR [12]. In both cases the transcription start sites are removed, likely resulting in the lack of the transcript. Accordingly to Peixoto et al. [7], BRCA2 c.156_157insAluYα5 is classified as deleterious since it results in exon 3 skipping and co-segregates with the disease. However, the pathogenicity of the duplication of BRCA1 exons 1-2 cannot be assessed. Despite the efforts carried out by del Valle et al. [12] and by us to study the effect at the DNA level we were unable to amplify the aberrant allele. Moreover, a co-segregation study could not be performed in none of the families identified in each report. Therefore, this variant must remain as of uncertain significance.

Homologous vs. non-homologous recombination as the origin of BRCA1/2 LGRs

Having identified non-homologous recombination events as the sources of three of the four BRCA2 LGRs they analyzed, Ruiz de Garibay et al. suggested that the proportion of BRCA2 LGRs originated by homologous recombination had been overestimated [14]. For BRCA1 LGRs, the present results are in keeping with statistics showing the predominant mechanism to be Alu-mediated homologous recombination [4]. Furthermore, all the Alu elements apparently involved in producing the BRCA1 LGRs observed in the present study are members of the evolutionarily youngest subfamilies, Alu\(\Sigma\) and Alu\(\Upsilon\), which have a high degree of mutual homology [29].

BRCA1/2 LGRs in the Iberian Peninsula

Founder mutations are responsible for the high rates of LGRs in the Valencian Community (Eastern Spain), where NG_005905:97346_111983del has deleted BRCA1 exons 3-5 in 10.97% of all families with mutations in the BRCA1 gene [30], and in Portugal, where BRCA2 c.156_157insAluYα5 (NG_012772:8686–8687ins AluYα5) accounts for 57.89% of all mutant BRCA2 families [8]. By contrast, in our population deleterious LGRs constitute only 2.34% (95% CI: 0.61–7.22) of all families with definitely pathogenic BRCA1 variants, given the small duplication exon1-2dup cannot be classified as a deleterious mutation. The observed frequency is the lowest rate reported to date for BRCA1 LGRs in an Iberian population. We should however note that given that most of the studies performed to date in Iberian populations are characterized by their limited sample size, the accuracy of the estimates is limited, as it is demonstrated by the wide interval of the frequency at 95% confidence level. Nonetheless, the frequency of BRCA1 LGRs within BRCA1 mutation carriers in Galician population seems to differ from Spanish (\(P\)-value = 0.013) and Iberian populations (\(P\)-value = 0.014).

Role of MLPA in testing for BRCA1/2 LGRs

MLPA is a fast, sensitive means of detecting LGRs, and cannot at present be replaced by massively parallel sequencing methods: recent studies suggest that these latter are adequate for detection of point mutations of BRCA1 and BRCA2, but are insufficiently specific for LGRs [31]. However, the optimization of next generation sequencing standard protocols for detection of Alu element rearrangements have resulted in false positive reads [32]. The shortcoming of MLPA is that it does not identify LGR breakpoints, as is necessary for recognition of recurrent rearrangements, for inference of the molecular mechanisms of rearrangement, and for rapid analysis of a proband’s relatives. Breakpoint identification still requires other strategies, such as

| Table 3. Frequency of BRCA1 and BRCA2 deleterious LGRs. |
|----------------------------------------------------------|
| Galicia | Spain* | Iberian population* | Galicia vs Spain | Galicia vs Iberian populations |
| N | % (95% CI) | N | % (95% CI) | N | % (95% CI) | P-value* | P-value* |
| NLGR | 3 | 2.34 (0.61–7.22) | 27 | 9.93 (6.76–14.27) | 28 | 9.76 (6.69–13.94) | 0.013 | 0.014 |
| NBRCA1 | 128 | 272 | 287 |

\(N_{\text{LGR}}\): number of families with LGRs; \(N_{\text{BRCA1}}\): number of families with BRCA1 mutations.

*Estimated from the reports performed in Spanish populations with available data [9,11,15,16].

**Estimated from the reports performed in Portuguese populations with available data [6,7,9,11,15,16].

\(P\)-value estimated from the reports performed in Iberian populations with available data [6,7,9,11,15,16].

Chi-square test with Yates correction \(P\)-value.

doi:10.1371/journal.pone.0093306.t003
methods based on Sanger sequencing. Another question is whether MLPA should be performed before or after screening for point mutations and small indels. Given the relatively high frequency of LGRs they found among BRCA1 for point mutations and small indels. Given the relatively high frequency of LGRs they found among BRCA1, and 2.34% of all families with BRCA1 mutations in Galicia, the lowest figure reported to date in the Iberian population. All three involve Alu elements, which corroborates the predominance of Alu-mediated mechanisms in the production of BRCA1 LGRs. LGRs affecting the same exons have been reported previously, but without breakpoint determination, and in some cases cannot have coincided with those observed in this study. We also detected one BRCA1 LGR, the Portuguese founder mutation c.156_157insAluYA5. To our knowledge, this is the first time this mutation has been detected in a family not known to be of Portuguese origin. However, a distant Portuguese ancestry cannot be ruled out.

Supporting Information

Figure S1 AluSq2 replacement by AluSx1 in NC_000017.10:g.41230935_41399840delInsAluSx1. a) Patient’s electropherogram. b) Reference sequence, patient sequence, and AluSx1 sequence (Repbase Sequences). c) Blastn suite. (DOCX)

Table S1 Primer sequences and PCR conditions. (DOCX)

Acknowledgments

We are grateful to all participants for their cooperation, and to Ines Quintela of the Spanish National Genotyping Centre (http://www.cegen.org) for her support as Alimetrix genotyping platform manager.

Author Contributions

Conceived and designed the experiments: AV. Performed the experiments: LF, AB. Analyzed the data: LF. Contributed reagents/materials/analysis tools: AC, MS. Wrote the paper: LF, AV.

References

1. Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, et al. (1994) A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science 266: 66–71.
2. Wooster R, Bignell G, Lancaster J, Swift S, Seal S, et al. (1995) Identification of the breast cancer susceptibility gene BRCA1. Nature 378: 789–792.
3. Anglian Breast Cancer Study Group (2000) Prevalence and penetrance of BRCA1 and BRCA2 mutations in a population-based series of breast cancer cases. Br J Cancer 83: 1901–1308.
4. Shiüler M, van Rensburg E (2011) Large genomic rearrangements of the BRCA1 and BRCA2 genes: review of the literature and report of a novel BRCA1 mutation. Breast Cancer Res Treat 125: 325–349.
5. Wahli T, Casadei S, Coats KH, Swhier E, Stray SM, et al. (2006) Spectrum of Mutations in BRCA1, BRCA2, CHEK2, and TP53 in Families at High Risk of Breast Cancer. JAMA 295: 1357–1360.
6. Peixoto A, Salgueiro N, Santos C, Varzim G, Rocha P, et al. (2006) BRCA1 and BRCA2 germline mutational spectrum and evidence for genetic anticipation in Portuguese breast/ovarian cancer families. Breast Cancer Res Treat 95: 379–387.
7. Peixoto A, Santos C, Rocha P, Pinheiro M, Príncipe S, et al. (2009) The c.156_157insAlu BRCA2 rearrangement accounts for more than one-fourth of deleterious BRCA2 mutations in northern/central Portugal. Breast Cancer Res Treat 114: 31–38.
8. Peixoto A, Santos C, Pinheiro M, Pinto S, Soares M, et al. (2011) International distribution and age estimation of the Portuguese BRCA2 c.156_157insAlu founder mutation. Breast Cancer Res Treat 127: 671–679.
9. de la Hoy a M, Gutiérrez-Enríquez S, Velasco E, Osorio A, de Abajo AS, et al. (2006) Genomic rearrangements at the BRCA1 locus in Spanish families with breast/ovarian cancer. Clin Chem 52: 1480–1485.
10. Gutiérrez-Enríquez S, de la Hoy a M, Martínez-Bouzas C, de Abajo A, Caijal T, et al. (2007) Screening for large rearrangements of the BRCA1 gene in Spanish families with breast/ovarian cancer. Breast Cancer Res Treat 103: 103–107.
11. Miram a M, Calvo M, Rodríguez A, Antón A, Lorente F, et al. (2008) Genetic analysis of BRCA1 and BRCA2 in breast/ovarian cancer families from Aragon (Spain): two novel truncating mutations and a large genomic deletion in BRCA1. Breast Cancer Res Treat 112: 353–358.
12. del Valle J, Feliubadó L, Nadal M, Teulé A, Miro R, et al. (2010) Identification and comprehensive characterization of large genomic rearrangements in the BRCA1 and BRCA2 genes. Breast Cancer Res Treat 122: 733–743.
13. Rodríguez M, Torres A, Borrás J, Saltat M, Gumà J (2010) Large genomic rearrangements in mutation-negative BRCA families: a population-based study. Clin Genet 78: 405–407.
14. Ruiz de Garibay G, Gutiérrez-Enríquez S, Garre P, Bonache S, Romero A, et al. (2012) Characterization of four novel BRCA1/BRCA2 large genomic rearrangements in Spanish breast/ovarian cancer families: review of the literature, and reevaluation of the genetic mechanisms involved in their origin. Breast Cancer Res Treat 133: 273–283.
15. Juan Jiménez I, García Casado Z, Palanca Suela S, Esteban Cardetéa E, López Guerrero J, et al. (2013) Novel and recurrent BRCA1/BRCA2 mutations in early onset and familial breast and ovarian cancer detected in the Program of Genetic Counseling in Cancer of Valencian Community (eastern Spain). Relationship of family phenotypes with mutation prevalence. Fam Cancer 1: 1–11.
16. Blay P, Santamaría I, Prieto A, Luque M, Alfredo M, et al. (2013) Mutational analysis of BRCA1 and BRCA2 in hereditary breast and ovarian cancer families from Asturias (Northern Spain). BMC Cancer 13: 243.
17. Palanca Suela S, Esteban Cardetéa E, Barragán González E, Oltra Soler S, de Juan Jiménez I, et al. (2008) Identification of a novel BRCA1 large genomic rearrangement in a Spanish breast/ovarian cancer family. Breast Cancer Res Treat 112: 63–67.
18. Fachal L, Rodríguez-Pazos L, Ginarte M, Toribio J, Salas A, et al. (2012) Multiple local and recent founder effects of TGMI in Spanish families. PLoS One 7: e33580.
19. Vega A, Campos B, Bressac-de-Pallerets B, Bond PM, Janin N, et al. (2004) The R716del BRCA1 is a founder Spanish mutation and leads to aberrant splicing of the transcript. Hum Mutat 17: 530–532.
20. Loidi L, Quirineiro C, Parajes C, Barreiro J, Lestón DG, et al. (2006) High variability in CYP21A2 mutated alleles in Spanish 21-hydroxylase deficiency patients, six novel mutations and a founder effect. Clin Endocrinol (Oxf) 64: 330–336.
21. Vega A, Torres M, Martínez J, Ruiz-Ponte C, Barros F, et al. (2002) Analysis of BRCA1 and BRCA2 in breast and breast/ovarian cancer families shows population substructure in the Iberian peninsula. Ann Hum Genet 66: 29–36.
22. Galician Society of Medical Oncology (2011) Galician Hereditary Cancer Guide; Oncology GSOM, editor.
23. MRC-Holland website. Available: www.mrc-holland.com. Accessed 2014 Mar 9.
24. RepeatMasker Open-3.0 website. Available: http://www.repeatmasker.org. Accessed 2014 Mar 6.
25. García-Casado Z, Romero I, Fernandez-Serra A, Rubio L, Llopis F, et al. (2011) A de novo complete BRCA1 gene deletion identified in a Spanish woman with early bilateral breast cancer. BMC Med Genet 12: 154.
26. Konceny M, Zavadka K, Vranova V, Vizvayrva M, Weimannova E, et al. (2008) Identification of rare complete BRCA1 gene deletion using a combination of SNP haplotype analysis, MLPA and array-CGH techniques. Breast Cancer Res Treat 109: 501–503.
27. Engert S, Wappenschmidt B, Beta B, Kast K, Kutsche M, et al. (2008) MLPA screening in the BRCA1 gene from 1,506 German hereditary breast cancer cases: novel deletions, frequent involvement of exon 17, and occurrence in single early-onset cases. Hum Mutat 29: 948–958.
28. Pylkas K, Erkko H, Nikkila J, Solyom S, Winqvist R (2008) Analysis of large deletions in BRCA1, BRCA2 and PALB2 genes in Finnish breast and ovarian cancer families. BMC Cancer 8: 146.
29. Gu W, Zhang F, Lupski JR (2008) Mechanisms for human genomic rearrangements. PahloGenetics 1: 4.
30. Palanca S, de Juan I, Pérez-Simó G, Barragán E, Chirivella I, et al. (2013) The deletion of exons 3–5 of BRCA1 is the first founder rearrangement identified in breast and/or ovarian cancer Spanish families. Fam Cancer 12: 119–123.
31. Feilhabadlo L, Lopez-Doriga A, Castellsague E, del Valle J, Menendez M, et al. (2012) Next-generation sequencing meets genetic diagnostics: development of a comprehensive workflow for the analysis of BRCA1 and BRCA2 genes. Eur J Hum Genet.

32. De Brakeleer S, De Grève J, Lissens W, Teugels E (2013) Systematic Detection of Pathogenic Alu Element Insertions in NGS-Based Diagnostic Screens: The BRCA1/BRCA2 Example. Hum Mutat 34: 785–791.

33. Hartmann C, John AL, Klaes R, Hofmann W, Bielen R, et al. (2004) Large BRCA1 gene deletions are found in 3% of German high-risk breast cancer families. Hum Mutat 24: 534–534.

34. Bunyan DJ, Eccles DM, Sillibourne J, Wilkins E, Thomas NS, et al. (2004) Dosage analysis of cancer predisposition genes by multiplex ligation-dependent probe amplification. Br J Cancer 91: 1155–1159.