

RESEARCH ARTICLE

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Analysis of *SLX4/FANCP* in non-*BRCA1/2*-mutated breast cancer families

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Abstract

Background: Genes that, when mutated, cause Fanconi anemia or greatly increase breast cancer risk encode for proteins that converge on a homology-directed DNA damage repair process. Mutations in the *SLX4* gene, which encodes for a scaffold protein involved in the repair of interstrand cross-links, have recently been identified in unclassified Fanconi anemia patients. A mutation analysis of *SLX4* in German or Byelorussian familial cases of breast cancer without detected mutations in *BRCA1* or *BRCA2* has been completed, with globally negative results.

Methods: The genomic region of *SLX4*, comprising all exons and exon-intron boundaries, was sequenced in 94 Spanish familial breast cancer cases that match a criterion indicating the potential presence of a highly-penetrant germline mutation, following exclusion of *BRCA1* or *BRCA2* mutations.

Results: This mutational analysis revealed extensive genetic variation of *SLX4*, with 21 novel single nucleotide variants; however, none could be linked to a clear alteration of the protein function. Nonetheless, genotyping 10 variants (nine novel, all missense amino acid changes) in a set of controls (138 women and 146 men) did not detect seven of them.

Conclusions: Overall, while the results of this study do not identify clearly pathogenic mutations of *SLX4* contributing to breast cancer risk, further genetic analysis, combined with functional assays of the identified rare variants, may be warranted to conclusively assess the potential link with the disease.

Background

A functionally coherent network of gene and/or protein interactions, altered in Fanconi anemia (FA) and breast cancer (BrCa), has emerged in recent years [1]. Fifteen genes that, when mutated, cause FA (*FANC* genes) and several genes that may harbor mutations of high, moderate or low penetrance for BrCa risk encode for proteins that converge on a homology-directed DNA damage repair process [2]. As further evidence of a fundamental common causal basis between these diseases, germline bi- and mono-allelic loss-of-function mutations

in four of these genes cause FA and BrCa, respectively: *FANCD1/BRCA2* [3,4], *FANCF/BRIP1* [5-8], *FANCN/PALB2* [9-11] and *FANCO/RAD51C* [12,13] (more recent data suggests that mutations in *RAD51C* may be primarily linked to ovarian cancer risk [14]). This evidence marks any novel gene involved in the aforementioned network or process as a candidate to harbor mutations in unclassified FA and/or BrCa patients.

Interstrand DNA cross-link agents, such as mitomycin-C used in diagnostic tests for FA, block replication forks and may therefore cause genome instability. Homologs of *SLX4* in model organisms were initially identified as necessary for replication fork restart following exposure to DNA-damaging agents [15]. Subsequently, *SLX4* homologs have been shown to play a key role as docking molecules for the repair of interstrand cross-links [16,17]. These observations pointed to the human *SLX4* gene as a *FANC* candidate for unclassified

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patients and, as a result, two groups have recently described mutations (renamed *FANCP* gene or FA-P subtype) [18,19]. The potential link to BrCa risk has been examined, to date, in 52 German or Byelorussian patients with familial breast cancer [20]: the study has not revealed truncating or clearly pathogenic mutations, but has identified four unclassified missense variants. Here, we conducted a more detailed study of the *SLX4* gene in 94 index BrCa cases from Spanish families negative for *BRCA1* and *BRCA2* mutations. As recently reported [20], our results do not show truncating or clearly pathogenic mutations, although they do describe seven missense variants of unknown biological significance that are not found in controls.

Methods

Study samples

Since its creation in 1999, the Hereditary Cancer Program at the ICO has identified a set of high-risk families with suspected hereditary breast and/or ovarian cancer syndrome. Following the Catalan Consensus Onco Guidelines on genetic testing for this condition, patients are analysed for mutations in the *BRCA1* or *BRCA2* genes after receiving appropriate genetic counselling and providing written informed consent. This genetic analysis consists of screening for point mutations and large rearrangements affecting those genes. For the present study, a total of 94 affected individuals belonging to 94 unrelated families negative for *BRCA1* or *BRCA2* mutations were selected. In addition to negativity for mutations in *BRCA1* or *BRCA2*, the inclusion criteria were: at least three first-degree relatives affected by breast or ovarian cancer; or at least two first-degree female relatives affected by breast cancer (at least one of them diagnosed before the age of 50); or at least one case of female breast cancer plus at least one case of either ovarian, female bilateral breast, or male breast cancer. Among the selected cases, 10 families were represented with an elevated prior risk of harboring a high-penetrance mutation, as calculated with the BRCAPRO [21] algorithm (briefly, 94 families mean = 0.40, standard deviation = 0.26, 95% confidence interval 0.11-0.99). Control samples, consisting of 138 women and 146 men, were taken from a hospital-based cancer association study (a detailed description of the study population, composition and interviews has been given elsewhere [22]). Specifically, these individuals were randomly enrolled from non-cancer patients admitted to the same general hospital as the BrCa cases. To avoid selection bias, the inclusion criterion for controls was that the current admission to the hospital should be for a new disease (not previously diagnosed). The studies were approved by the IDIBELL ethics committee and participants gave

written informed consent for their participation, and for the genetic analysis of their biological samples, according to the Declaration of Helsinki.

Mutation analysis

The *SLX4* exons and exon-intron boundaries were sequenced from polymerase chain reactions using previously defined primers and conditions [18]; for exon 7, AmpliTaq Gold DNA Polymerase (Applied Biosystems) and 10% of dimethyl sulfoxide were used. The reaction products were purified from remaining primers using ExoSAP-IT (GE Healthcare) and sequencing reactions performed following a standard Sanger method with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Samples harboring DNA variants were re-sequenced at least once using an independent DNA aliquot from the first-pass analysis. The guidelines of the Human Genome Variation Society and the reference sequences NM_032444.2 and NP_115820.2 of the National Center for Biotechnology Information were used for nomenclature.

Genotyping

Assays based on the KASPar technology were performed following the manufacturer's instructions (KBioscience). Reactions were carried out in a 384-well format with 2% of duplicates, and negative and positive (i.e. BrCa patient carrier) sample controls present in each plate.

Results and discussion

The genomic region of *SLX4*, comprising all exons and exon-intron boundaries, was sequenced in 94 BrCa familial cases that match a criterion indicating the potential presence of a highly-penetrant germline mutation, following exclusion of *BRCA1* or *BRCA2* mutations (see Methods). This mutational analysis revealed 49 variants: 21 novel and 28 which are currently annotated in the single nucleotide polymorphism database (dbSNP [23]) (Table 1). Of the 49 variants, 21 were found only once, which include three changes identified by the 1,000 Genomes Project [24] (Table 1): rs72778139-T has no known frequency data; rs76488917-A has an allele frequency of 0.02 in Caucasians; and rs115694169-A has an allele frequency of 0.03 in the Yoruba people of Ibadan (there is no data for Caucasians). Excluding these from the set of 21 with low frequency revealed eight missense and five silent changes at the protein level, and five intronic changes (Table 1). A neural network splicing prediction [25] model did not strongly support alteration by any of the identified intronic variants (data not shown). Together, these results suggest extensive genetic variation at the *SLX4* locus among individuals in our population, but provide no obvious link to BrCa risk.

Table 1 SLX4 variants found in non-BRCA1/2-mutated familial BrCa cases

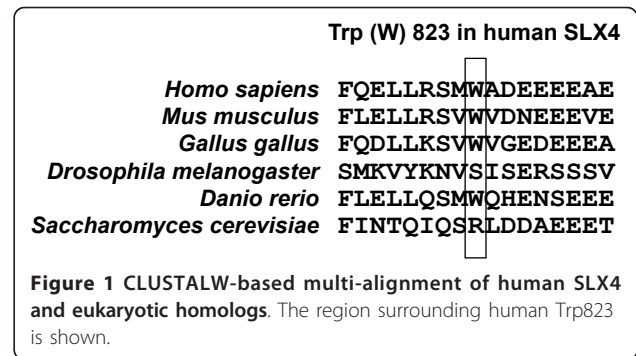
Exon	Nucleotide change	Change type	Amino acid change	Number of carriers*			dbSNP†
				Het (%)	Hom (%)	Total	
2	c.248G > C	Missense	p.Gly83Ala	1 (1.1)	0	94	NA
2	c.339T > C	Silent	p. =	1 (1.1)	0	94	NA
2	c.421G > T	Missense	p.Gly141Trp	1 (1.1)	0	94	NA
3	c.555C > T	Silent	p. =	9 (9.5)	0	94	rs74640850
3	c.610C > T	Missense	p.Arg204Cys	10 (10.6)	0	94	rs79842542
3	c.678C > T	Silent	p. =	3 (3.2)	0	94	rs28516461
3	c.590T > C	Missense	p.Val197Ala	1 (1.1)	0	94	NA
3	c.710G > A	Missense	p.Arg237Gln	2 (2.1)	0	94	NA
3	c.753G > A	Silent	p. =	24 (25.5)	2 (2.1)	94	rs8061528
4	c.761-32T > G	Intronic	p. =	2 (2.1)	0	94	NA
5	c.1152A > G	Silent	p. =	11 (11.7)	0	94	rs112511042
5	c.1153C > A	Missense	p.Pro385Thr	1 (1.1)	0	94	rs115694169
5	c.1156A > G	Missense	p.Met386Val	11 (11.7)	0	94	rs113490934
5	c.1163 + 10C > T	Intronic	p. =	11 (11.7)	0	94	rs80116508
6	c.1164-16T > C	Intronic	p. =	1 (1.1)	0	94	NA
6	c.1164-40C > A	Intronic	p. =	1 (1.1)	0	94	NA
6	c.1164-66T > A	Intronic	p. =	2 (2.1)	0	94	NA
6	c.1164-75C > G	Intronic	p. =	11 (11.7)	0	94	rs59622164
6	c.1366 + 11T > C	Intronic	p. =	12 (12.8)	0	94	rs76350200
7	c.1371T > G	Missense	p.Asn457Lys	10 (10.6)	0	94	rs74319927
7	c.1419C > T	Silent	p. =	1 (1.1)	0	94	NA
8	c.1846G > A	Missense	p.Val616Met	1 (1.1)	0	94	NA
9	c.2012T > C	Missense	p.Leu671Ser	11 (11.8)	0	93	rs77985244
9	c.2013 + 23G > A	Intronic	p. =	11 (11.7)	0	94	rs112226642
9	c.2013 + 137G > C	Intronic	p. =	11 (11.7)	0	94	rs80186343
10	c.2160 + 50C > T	Intronic	p. =	10 (10.6)	0	94	rs75762935
12	c.2346C > T	Silent	p. =	1 (1.1)	0	94	NA
12	c.2469G > C	Missense	p.Trp823Cys	1 (1.1)	0	94	NA
12	c.2854G > A	Missense	p.Ala952Thr	8 (8.5)	0	94	rs59939128
12	c.2855C > T	Missense	p.Ala952Val	8 (8.5)	0	94	rs78637028
12	c.3162G > A	Silent	p. =	1 (1.1)	0	94	rs76488917
12	c.3365C > T	Missense	p.Pro1122Leu	12 (12.8)	1 (1.1)	94	rs714181
12	c.3662C > T	Missense	p.Ala1221Val	10 (10.6)	0	94	rs3827530
12	c.3812C > T	Missense	p.Ser1271Phe	4 (4.2)	0	94	rs3810813
12	c.3872C > T	Missense	p.Thr1291Met	1 (1.1)	0	94	NA
12	c.4261A > T	Missense	p.Ile1421Phe	1 (1.1)	0	94	NA
12	c.4409C > T	Missense	p.Pro1470Leu	1 (1.1)	0	94	rs72778139
12	c.4500T > C	Silent	p. =	42 (44.7)	21 (22.3)	94	rs3810812
12	c.4530G > T	Silent	p. =	1 (1.1)	0	94	NA
13	c.4637-125C > T	Intronic	p. =	1 (1.1)	0	94	NA
13	c.4637-227C > T	Intronic	p. =	9 (9.6)	0	94	rs75693937
13	c.4739 + 10C > T	Intronic	p. =	1 (1.1)	0	94	NA
13	c.4739 + 24G > T	Intronic	p. =	20 (21.3)	2 (2.1)	94	rs12933120
14	c.5072A > G	Missense	p.Asn1691Ser	1 (1.1)	0	94	NA
15	c.5389C > T	Silent	p. =	1 (1.1)	0	93	NA
15	c.5501A > G	Missense	p.Asn1834Ser	2 (2.2)	0	93	rs111738042
15	c.*8A > G	Intronic	p. =	9 (9.7)	0	93	rs3751839
15	c.*102G > A	Intronic	p. =	1 (1.1)	0	93	NA
15	c.*113C > T	Intronic	p. =	8 (8.6)	0	93	rs76661336

*Het, heterozygous; Hom, homozygous

†Build 133; NA, not applicable

Having identified rare variants in BrCa familial cases, we next assessed the presence of 10 of these variants in a cohort of controls collected at the same hospital as the cases (see Methods). The selection of these variants was based on the observed low frequency in the 94 BrCa cases and on their identification as missense variations. In addition, a causative prediction was obtained using two algorithms (PolyPhen-2 [26] and SIFT [27]), plus a weighted average of scores (Condel [28]). Seven of these variants were not found in controls and, intriguingly, five of them were predicted to be “deleterious” (Table 2). Among this group, only one amino acid position (Trp823) showed some evolutionary conservation (Figure 1), and the substitution may be disfavored (Trp to Cys) [29]; tumor samples were not available for any case that would have allowed assessment of the existence of loss of heterozygosity at the *SLX4* locus. Nonetheless, predictions of a deleterious effect should be taken with caution as neutral polymorphisms can frequently be misclassified (from ~15-50% depending on the method [28]). On the other hand, extensive genetic variation in *SLX4* might reflect an unknown evolutionary pressure or could be related to a similar observation made for other DNA repair-related genes [30].

While *SLX4* serves as a scaffold for multiple proteins involved in the DNA damage response [16,17], the functional involvement of the Trp823 position, and of the other rare variants not found in controls in this study, is unknown. None of the identified variants changes a



critical amino acid residue and there is no data that could suggest an alteration of protein interactions or complexes; however, the Pro1470Leu variant might disrupt a mitotic phosphorylation site at Ser1469 [31,32]. In this context, a *SLX4* pathological variant linked to BrCa should consist of a hypomorphic mutation that would cause genome instability. Accordingly, *SLX4* is a key regulator of the function of structure-specific endonucleases involved in the repair of DNA damage; in particular, proper function of *SLX4* is fundamental for repair during replication and for resolving Holliday junctions formed during homologous recombination [16,17,33,34]. Overall, the results of this study do not support the existence of loss-of-function mutations of *SLX4* associated with BrCa risk; nonetheless, further genetic analysis in patients and controls, combined with

Table 2 Pathological prediction and frequency in controls of selected *SLX4* missense variants

Exon	Nucleotide change	Amino acid change	dbSNP†	Pathological prediction				Controls tested (n)	Number of control carriers (%)
				SIFT (score < 0.05, deleterious)	PolyPhen-2 (false positive rate)	Condel (weighted average of scores)	Condel prediction		
2	c.248G > C	p.Gly83Ala	NA	0.14	0.15	0.15	Neutral	283	0
2	c.421G > T	p.Gly141Trp	NA	0.00	0.86	0.86	Deleterious	284	2 (0.7)
3	c.590T > C	p.Val197Ala	NA	0.48	0.01	0.00	Neutral	284	1 (0.4)
3	c.710G > A	p. Arg237Gln	NA	0.49	0.00	0.38	Neutral	284	4 (1.4)
8	c.1846G > A	p. Val616Met	NA	0.17	0.62	0.80	Deleterious	281	0
12	c.2469G > C	p. Trp823Cys	NA	0.01	1.00	0.97	Deleterious	282	0
12	c.3872C > T	p. Thr1291Met	NA	0.05	0.98	0.99	Deleterious	283	0
12	c.4261A > T	p. Ile1421Phe	NA	0.08	0.77	0.77	Deleterious	285	0
12	c.4409C > T	p. Pro1470Leu	rs72778139	0.02	0.99	0.96	Deleterious	283	0
14	c.5072A > G	p. Asn1691Ser	NA	0.56	0.00	0.01	Neutral	285	0

†Build 133; NA, not applicable

functional assays of specific rare variants, may be warranted.

Conclusions

The mutational analysis of *SLX4* in 94 familial BrCa index cases without mutations in *BRCA1* or *BRCA2* has revealed extensive genetic variation. Twenty-nine novel single nucleotide variants have been detected, 21 of them showing relatively low allele frequencies: however, none can be linked to a clear alteration of the protein function. Nonetheless, analysis of 10 of these variants failed to detect seven of them in a set of controls. While the results of this study do not support the common existence of *SLX4* mutations contributing to BrCa risk, additional studies may be warranted.

Abbreviations

BrCa: Breast cancer; *BRCA1/2*: Breast cancer 1/2: early onset genes; BRCAPro: *BRCA* carrier prediction model; Condel: Consensus deleteriousness score; dbSNP: Single nucleotide polymorphism database; DNA: Deoxyribonucleic acid; FA: Fanconi anemia; Het: Heterozygous; Hom: Homozygous; PolyPhen: Polymorphism phenotyping; SIFT: Sorting intolerant from tolerant; *SLX4*: Structure-specific endonuclease subunit homolog (*S. cerevisiae*) gene.

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Authors' contributions

The project was conceived by DS, CL and MAP. The experiments and data analyses were coordinated by JF-R, CL and MAP. The mutational analysis was performed by JF-R and FQ. The clinical and *BRCA1/2* annotations were performed by IB, AT, LF, JV, MS, AI, ED, GC, JB and CL. The selection of index cases was performed by IB. The manuscript was written by MAP. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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