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A 7 Mb region within 11q13 may contain a high penetrance gene for breast cancer

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Abstract Familial breast cancer represents up to 5% of all breast cancer cases. Recently, our group has performed a new SNP-based linkage study in 19 non-*BRCA1/2* families. We found that a single family was linked to regions in two different chromosomes (11q13 and 14q21), and observed a non-parametric LOD score of 11.5 in both regions. In the present study, we ruled out any possible translocation between the chromosomes. We also used both a panel of STRs and an indirect approach based on Hap-Map data to narrow down these regions from 28 to 7 Mb in

chromosome 11 and from 14.5 to 8.5 Mb in chromosome 14. We performed a mutational screening on candidate genes in 11q13 (*NUMA1*, *FGF3*, *CCND1*, *RAD9A*, *RNF121*, *FADD* and *hsa-mir-192*), and on *FOXA1* in 14q21. Although we have not found any deleterious mutations in the coding region of these genes, data from STR markers confirm 11q13 as a candidate region to contain a breast cancer susceptibility gene.

Keywords Breast cancer · Linkage study · Haplotype · Singlepoint LOD score

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Introduction

Familial breast cancer represents up to 5% of all breast cancer cases [1]. The two known high-penetrance genes for breast cancer, *BRCA1* and *BRCA2*, are involved in about 25% of these families [2–5]. The remaining families do not carry mutations in these genes and are known as BRCAX families.

While several candidate regions to carry a susceptibility gene for breast cancer have been identified by linkage studies based on microsatellite (STR) [6–8] or SNP markers [9], no high-penetrance gene has been found since *BRCA1* and *BRCA2* were cloned.

There are a number of possible explanations for the lack of consistency in these results. One is the high heterogeneity within BRCAX cases suggested by some authors [10] and described by immunohistochemical studies [11]. Another is that susceptibility loci might be population-specific. Recently, our group performed a new SNP-based linkage study in 19 BRCAX families from Spain, The Netherlands and USA. In addition to demonstrating that SNPs have more power than STRs for linkage detection

[12], five candidate regions were described on chromosomes 2, 4, 7, 11 and 14, consistent with the existence of high heterogeneity within BRCAX families. A single family (FAM153) was linked to two of these regions (11q12.3q14.3 and 14q13.1q21.3), with a multipoint parametric LOD score under a dominant model (multipoint HLOD score) of 2.20, and non-parametric LOD scores of 11.50 (multipoint) and 4.50 (two points), in each region. These high LOD scores may be due to the fact that this family (FAM153) is large and very informative.

In the present study, we have performed a mutational screening of literature-based candidate genes in both these regions in this family. Although we have not found any mutations in the coding region of the genes studied, we have narrowed down the boundaries for both candidate regions and described different polymorphisms in both coding and non-coding regions.

Materials and methods

Familial cases and controls

The familial structure of FAM153 is shown in Fig. 1. FAM153 is a large family consisting of eighteen members from four generations, with six females affected with breast cancer. The family members available for the genotyping analysis were II-7, IV-1, IV-2 and IV-3.

Index cases from 125 high-risk breast cancer families (BRCAX) recruited through the CNIO were included to evaluate the frequency of rs11278712 (see Gene study section). These high-risk families contained at least two first-degree relative females affected with breast cancer (at least one of them diagnosed before 50), or at least three affected women of more distant kinship. DNA extracted from peripheral blood from the 125 index cases had been fully screened for mutations in *BRCA1* and *BRCA2* by a combination of DHPLC and direct sequencing, without detecting any deleterious mutations.

In addition, DNA samples from 465 non-related Spanish women aged between 40 and 65 and without personal or familial antecedents of any type of cancer were analyzed to establish the frequency of rs11278712 in the general population.

Candidate regions

The candidate region on chromosome 11 identified in our previous study [12] spans from rs1525064 to rs1388472, or over 28 Mb (Fig. 1a), while that on chromosome 14 spans from rs2027338 to rs1532202, covering around 14.5 Mb (Fig. 1b). Both regions gave a multipoint HLOD score of 2.20 [12].

In order to confirm and narrow down these regions, we performed a fine-mapping study based on STRs. We genotyped a total of 11 STRs (D11S4174, D11S4191, D11S4076, D11S1889, D11S4136, D11S4081, D11S4172, D11S901, D11S4147, D11S1367 and D11S873) covering and flanking the candidate region in chromosome 11 and 6 STRs (D14S1034, D14S70, D14S1014, D14S129, D14S288 and D14S269) covering and flanking the candidate region in chromosome 14 (Fig. 1). We calculated the singlepoint HLOD score for each marker using Merlin software [13].

Significance testing

We estimated the empirical probability of obtaining two peaks with multipoint HLOD >2.20 in the same family by chance alone, by performing a simulation study based on our set of 19 BRCAX families [12]. This was carried out using Merlin software.

The probability that two independent loci segregate together in a family was calculated assuming that these two loci came from an unknown single ancestor, as:

$$p = 2 \times (1/4)^n, \text{ where } n \text{ is the total number of meioses.}$$

Cytogenetic and FISH analyses

In order to rule out the possibility that a germinal translocation between chromosomes 11 and 14 segregated in FAM153, two analyses were performed on peripheral blood cells from one member of the family: a conventional GTG-banding technique, and FISH analysis. The FISH analysis was performed using DNA probes specific for the subtelomere of the long arm of chromosome 14 (GS-820M16, red) and the centromere of chromosome 11 (CEP11, green, Vysis, Inc. Downers Grove, IL, USA), and DNA probes specific for the subtelomere of the long arm of chromosome 11 (GS-26N8, red) and the short arm of chromosome 11 (GS-908H22, green) in order to exclude a cryptic translocation $t(11;14)$. These techniques were performed according to the standard procedures in our laboratories.

HapMap-based haplotype study

Due to the large size of the haplotypes shared by the four members studied in FAM153 (28 Mb for 11q13 and 14.5 Mb for 14q21), we sought to narrow down the region using an approach to identify possible recombination spots in the chromosomes in earlier generations. This approach was based on the idea that the haplotype shared by this family is the result of recombination events that occurred between the ancestral chromosome and other chromosomes,

Fig. 1 Representation of the HLOD score and the common haplotypes shared by FAM153. *Shadowed light green regions* represent the blocks selected in each chromosome using the indirect approach based on HapMap data. *Shadowed dark green boxes* represent the location for maximum singlepoint values.

a Representation of the region on chromosome 11.

b Representation of the region on chromosome 14

Fig. 2 Representation of the indirect approach using HapMap data to narrow down the candidate regions. **a** Theoretical representation of the approach. The ancestral haplotype (*Hap Ori*) showing putative recombination spots (*RS*) and the putative marker linked to the causal mutation (*grey box*). Different groups of haplotypes imputed from the Caucasian population (*Hap Pop N*) are used to identify the possible recombination spots. The derived haplotype (*Hap Aff*) shows different blocks of SNPs (estimated based on the putative recombination spots by comparing to HapMap haplotypes) and the marker linked to the causal mutation (*grey box*). **b** Diagram used to identify the blocks of

SNPs within the region in chromosome 11. The affected haplotype is divided into four blocks. There are four different groups of HapMap haplotypes (one representative haplotype is shown for each group) used to obtain three putative recombination spots (*RS*). The *white box* represents the location of the maximum singlepoint LOD score (*D11S4136*). *rs477238* and *rs514933* mark the limits of the minimal candidate region. **c** Diagram for the region on chromosome 14. The *white box* represents the location of the maximum singlepoint LOD score (*D14S129*). *rs1168539* and *rs1381641* mark the limits of the minimal candidate region

through the generations, since the disease allele appeared in the germ line of a common ancestor (Fig. 2a). It was based on HapMap data from 30 Caucasian trios (http://hapmap.org/cgi-perl/gbrowse/hapmap_B35/) for all the SNP markers in the candidate region that are included in the Illumina Linkage Panel III. We used these haplotype data to select the most probable combination of haplotypes that shared haplotype blocks with our putative risk haplotypes. We then selected the haplotype blocks that contained the STRs that showed the maximum singlepoint LOD scores in our candidate regions in chromosomes 11 and 14 (Fig. 2b, c). The aim of this approach was to obtain the minimal regions from the ancestral chromosomes carried through to the observed chromosomes, assuming the limitations that have been reported to be intrinsic to the estimation of haplotypes using HapMap data for a group of SNPs not in strong LD (limited number of chromosomes, missing genotypes, double heterozygotes and double recombination events).

Gene selection

Although we reduced the size of the candidate regions, the high number of loci within them forced us to establish two criteria for the selection of genes on which to performing mutational screening: (a) close proximity to the maximum

singlepoint LOD score, and (b) previous publication of function-, expression- and/or cancer-related information. This information was obtained from the NCBI database (<http://www.ncbi.nlm.nih.gov/sites/entrez>). The selected genes and their functional descriptions are summarized in Table 1. We firstly selected *NUMA1* in chromosome 11 and *FOXA1* in chromosome 14 as candidate genes. Five further genes (*CCND1*, *FADD*, *FGF3*, *RNF121*, and *RAD9A*) and one microRNA (*hsa-mir-192*, information extracted from <http://microrna.sanger.ac.uk/cgi-bin/targets/v3/search.pl>) were selected within the 11q13 region.

Gene study

For all the selected genes, mutational screening was carried out by direct sequencing on an ABI Prism 3700 sequencer. Reference sequences for all coding and UTR regions of the genes were obtained from the Genome database (www.ensembl.org). Primers were designed with at least 20 bp from flanking intronic sequences using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The sequencing was performed on DNA from individual IV-1 using the forward oligonucleotide, and where necessary (for example, where intron-exon boundaries were not well-defined), the sequencing was repeated using the reverse oligonucleotide. When a rare variant in the sequence was

Table 1 Description of the sequenced genes in the candidate regions in chromosomes 11 and 14

Chromosome	Location	Gene	Position	Function
11	11q13.1	<i>hsa-mir-192</i>	64415185	MicroRNA supposed to regulates cancer-related genes
	11q13.1-q13.2	<i>RAD9A</i>	66915999	Cell cycle checkpoint protein required for cell cycle arrest and DNA damage repair in response to DNA damage
	11q13.3	<i>CCND1</i>	69165054	Member of the cyclin family and mutations, amplification and overexpression of this gene are observed frequently in a variety of tumors and may contribute to tumorigenesis
	11q13.3	D11S4136	69324892	Microsatellite which shows maximum singlepoint value
	11q13.3	<i>FGF3</i>	69333917	Member of the fibroblast growth factor family and the closest gene to D11S4136
	11q13.3	<i>FADD</i>	69726917	Adaptor molecule that interacts with various cell surface receptors and mediates cell apoptotic signals
	11q13.4	<i>RNF121</i>	71317731	Gene with unknown function which contains a Ring Finger Domain supposed to be involved in DNA–protein and protein–protein interactions
	11q13.4	<i>NUMA1</i>	71391559	Mitotic checkpoint protein previously associated with breast cancer
14	14q12–q13	<i>FOXA1</i>	37128947	Transcription factor assumed to be a BRCA1 partner
	14q21.1	D14S129	38142033	Microsatellite which shows maximum singlepoint value

STR markers from the fine mapping strategy that showed significant singlepoint LOD scores are shown in bold

found, PCR amplification was repeated and checked for the four members of the family.

All variants found in the selected genes had been previously described in the databases (Ensembl, NCBI), with the exception of an indel of 6 bp within intron 12–13 of *NUMA1* (rs11278712), which had no allelic frequencies described in any population.

In order to estimate the allelic frequency of this variant and to assess its effect, we genotyped it in 465 controls and 125 BRCAX probands using DHPLC. To confirm the genotypes of the samples, we sequenced four subjects from each DHPLC profile.

Additional microsatellites in 11q13

We divided the candidate region in chromosome 11 using the indirect approach based on HapMap data. We selected the block defined between rs477138 and rs514933 based on the location of D11S4136, which showed the maximum singlepoint value (Fig. 3). In order to confirm this region as the most probable candidate region, we used a set of three STRs with high heterozygosity. We selected D11S987 (at 67.64 Mb, *het* = 0.82), D11S4113 (at 68.52 Mb, *het* = 0.81) and D11S4139 (at 70.18 Mb, *het* = 0.87) (Fig. 3). The allelic frequencies of these STRs in our population were calculated by genotyping 91 healthy controls (a total of 178 chromosomes for each marker).

We performed parametric analysis based on the markers previously studied plus these three additional STRs using Merlin software.

Results

Significance testing

In our previous linkage study [12], we found that a single family showed suggestive linkage signals in two different chromosomes, 11 and 14. We estimated the empirical probability of obtaining two peaks with multipoint HLOD >2.20 for a single family by chance to be 0.024. We estimated the probability that two independent loci could segregate in this family, with ten meioses, to be 0.000002.

Because finding two regions with high multipoint HLOD segregating in a single family is not expected by chance, we tested for a possible translocation between chromosomes 11 and 14 using both conventional karyotyping of G-bands and FISH analysis. Both analyses suggested that there was not a translocation between the two chromosomes (Fig. 4).

Candidate regions

We used a panel of 11 STRs in chromosome 11 and 6 STRs in chromosome 14 in order to confirm and narrow down the candidate regions. We also calculated the singlepoint HLOD score for each of these STRs, and obtained maximum singlepoint HLOD scores of 1.32 for D11S4136 in chromosome 11 and 1.33 for D14S129 in chromosome 14 (Fig. 1).

We used an indirect approach with HapMap data to narrow down the candidate regions (see section “[Materials and methods](#)” and Fig. 2a). We expected to observe a large

Fig. 3 Results from the fine mapping of the 11q13 region with STRs. The columns show the markers, physical distance (Mb), multipoint parametric LOD scores, singlepoint parametric LOD scores and genes sequenced. Markers that represent the limits of the region, obtained by the HapMap-based strategy, are shaded in *dark grey*. The original fine mapping markers are *underlined*. Additional markers used to confirm the reduced candidate region (*shaded*) are shown in *italics* and identified with an *arrow*. Markers with significant singlepoint LOD scores are shown in *bold*

Fig. 4 Cytogenetic analyses used to rule out any cryptic translocation between chromosomes 11 and 14. **a** Conventional GTG-banding of chromosomes 11 and 14 shows a normal structure for both chromosomes. **b** FISH analysis using probes specific for the 11q subtelomere (red) and 11 centromere (green). **c** FISH analysis using probes specific for the 11p subtelomere (green) and 11q subtelomere (red)

number of different haplotypes formed by SNPs in linkage disequilibrium blocks. The study of 30 CEPH trios showed a total of 167 different haplotypes for the region in chromosome 11 (formed by 38 SNPs), and 256 (formed by 26

SNPs) for the region in chromosome 14, and none of them were identical to the corresponding segregating risk haplotypes. We identified 4 LD blocks of SNPs in chromosomes 11 and 14, and for each chromosome, we

selected the block in which the marker with the maximum singlepoint LOD scores was located (Fig. 2b, c).

With this approach, we were able to narrow down the candidate region in chromosome 11 from 28 Mb (424 genes) to 7 Mb (202 genes, from rs477138 to rs514933). On the other hand, the candidate region in chromosome 14 which originally spanned 14.5 Mb (90 genes) was narrowed to 8.3 Mb (38 genes, from rs1168539 to rs1381641) (see Fig. 1). This reduction in the size of the candidate regions allows for more directed and affordable mutational screening.

Candidate gene sequencing

According to the criteria described in “Materials and Methods”, we first selected *NUMA1* and *FOXA1* as candidate genes for sequencing in the index case of FAM153. *NUMA1* is located on 11q13.4 (see Table 1) and *FOXA1* is located on 14q12–q13 (see Table 1). The sequencing of the coding region of *NUMA1* and *FOXA1* showed no new variation (supplementary table 1) from the reference sequence in the databases (Ensembl, NCBI). The only exception to this was an indel of 6 bp identified within intron 12–13 of *NUMA1* (rs11278712), for which no allelic frequencies were described in the databases.

To assess whether the derived allele was a polymorphism or a deleterious mutation, we examined its segregation in FAM153, and we confirmed its co segregation with breast cancer disease status (Table 2). Then, we genotyped this indel 465 control samples, and found 42 samples carrying the insertion allele, given an estimated minor allele frequency of 0.045. This result indicates that the derived allele is not a high-penetrance mutation. We extended this study to the probands from 125 BRCAX families and we found that the minor allele frequency was 0.028, very similar that among controls ($P = 0.24$).

Further genetic screening of 11q13

After the sequencing of *NUMA1* and *FOXA1*, we focused our efforts on the mutational screening of the 11q13 region. The main reason for this was the observed segregation of the indel allele with breast cancer in the family, suggesting the existence of a causal gene close to *NUMA1*. In addition, we found familial segregation of a set of rare alleles for three other SNPs within *NUMA1*: rs642573, rs1057992 and rs1541304 (see Table 2).

Following the selection criteria established in the material and methods section, we selected another five genes and one miRNA close to D11S4136, all within the 7 Mb region (Table 1; Fig. 3). We sequenced the coding and UTR regions of *FGF3*, *CCND1*, *FADD* and *hsa-mir-192*, but no new variation was found (supplementary table 1).

Table 2 List of the most infrequent SNPs found by sequencing in the candidate genes considered

Gene	RAD9A	RNF121	NUMA1			
SNP	rs11575914	rs1055452	rs3829215	rs1541306	rs642573	rs11278712
IV1 Genotype	A/C	C/T	C/T	A/G	C/G	-/GTCAAC
IV2 Genotype	C/C	C/T	C/T	A/G	C/G	-/GTCAAC
IV3 Genotype	C/C	C/T	C/T	A/G	C/G	-/GTCAAC
II7 Genotype	C/C	C/T	C/T	A/G	C/G	-/GTCAAC
European MAF	Freq(A) = 0.028	Freq(T) = 0.058	Freq(T) = 0.060	Freq(G) = 0.069	Freq(G) = 0.067	Freq(GTCAAC) = 0.045
						Freq(T) = 0.059
						Freq(A) = 0.058

An infrequent haplotype, consisting of seven low-frequency alleles (in bold) spread along RNF121 and NUMA1, is shared by all the individuals genotyped from family FAM153

We also selected *RNF121* to be sequenced because its functional domains predict a protein–DNA, protein–protein interacting molecule and it is located on the same LD block that *NUMA1* (data not shown). Although we did not find any new variation in the coding region of *RNF121*, we observed that this family shared a group of very infrequent alleles for seven variants found in *NUMA1* and *RNF121* (see Table 2), three of them in intronic regions of *NUMA1* (rs642573, rs1057992 and rs11278712), one of them in the 3'UTR regions of *NUMA1* (rs1541304) and other three in the 3'UTR of *RNF121* (rs1055452, rs3829215 and rs1541306) (see Table 2).

We genotyped these seven rare variants in five control samples that were heterozygous for the indel allele. We found that this rare haplotype likely appeared in four of these five controls. Thus, we estimated the minimum frequency of this haplotype to be greater than 3.6% in the general population. We therefore ruled out that this haplotype is deleterious for familial breast cancer.

As a final step in the mutational screening within 11q13, we sequenced *RAD9A*, but we did not find any new variation. However, we found that the index case of the family carried the minor A allele for rs11575914, which is described as having a frequency of 0.028 in the general population. We genotyped this SNP in the four individuals of the family, and found that the A allele did not segregate with breast cancer (see Table 2).

Confirmation of the 7 Mb candidate region (from rs477138 to rs514933)

In order to confirm the segregation of this region in family FAM153, we genotyped three STRs close to D11S4136 (Fig. 3). We observed that the allelic segregation of D11S987, D11S4113 and D11S4139 was consistent with the haplotype being shared by all genotyped members in the family. We then performed a parametric analysis using the data from our previous linkage study [12], and found that D11S987 and D11S4139 had even higher singlepoint values than D11S4136, which confirms that this region is the most likely candidate to carry the disease locus (Fig. 3).

Discussion

In the present study we have extended our previous work and described in detail two candidate regions to carry a breast cancer susceptibility gene. One region is located on chromosome 11 and spans 7 Mb. The other region is located on chromosome 14 and spans 8.5 Mb. Moreover, several genes within the 11q13 and 14q12–q21 regions have been screened but no deleterious mutations have been found. It should be noted that we cannot rule out the possibility of mutations

affecting the regulation of these genes (e.g., in promoter region) or large rearrangements, because sequencing was confined to the coding regions only.

Family FAM153 showed suggestive linkage to two peaks in two different chromosomes. We performed a simulation study in our set of families to estimate empirically the probability of finding a family with suggestive linkage in two different chromosomes, by chance along. This was statistically significant ($P = 0.024$), thus we assumed that two different high-risk loci could cosegregate in this family. In addition, we estimated the probability the two independent loci segregate together through the ten meioses observed in this family, and it was very low (1/524,288). However, some cases of familial segregation of mutations in both *BRCA1* and *BRCA2* have been reported [14, 15], highlighting that the transmission of mutations in two different and independent high-penetrance genes through multiple generations in a single family is possible, although very infrequent. Finally, we assessed whether a germinal translocation between both chromosome 11 and chromosome 14 exists, which could explain the familial segregation of these two regions, and the results confirmed the absence of any such translocation (Fig. 4).

Each of these two regions could therefore contain a high-risk breast cancer gene. Since we have not found any mutations in the genes considered, we cannot rule out that only one or neither of them contains a causal gene. We similarly cannot exclude the possibility that two moderate penetrance genes at each locus may interact, either with each other, or each with other low penetrance variants.

Due to the large size of the candidate regions (28 Mb in chromosome 11 and 14.5 Mb in chromosome 14), we applied both a fine mapping strategy based on STRs and an indirect approach based on HapMap data to narrow down these regions. The markers with the highest associated singlepoint LOD scores were the microsatellites, because they present greater heterozygosity than SNP markers and are therefore more informative. Using this strategy, we established that the maximum singlepoint LOD score was for D11S4136 in chromosome 11 and for D14S129 in chromosome 14 (Fig. 1). In addition, we applied an indirect approach to narrow down the affected shared region in both chromosomes using HapMap data from the Caucasian population (see section “Materials and Methods”).

We first selected *NUMA1*, in chromosome 11, and *FOXA1*, in chromosome 14, as candidate genes for sequencing. *NUMA1* has been previously described as a putative tumor suppressor gene associated with breast cancer and *FOXA1* belongs to an extensive transcription factor family spread across the genome (Table 1). The sequencing did not reveal any deleterious germline mutations in the coding regions of these genes, with the exception of an insertion of six base pairs (rs11278712) in

NUMA1 that was subsequently found to segregate in the family. Although we have ruled out its possible role as a high penetrance mutation, the presence of this marker segregating with breast cancer suggests that this chromosomal region may contain a putative causal gene. We therefore focused our efforts on further mutational screening of region 11q13.

Region 11q13 is considered to be important in cancer development, and specifically in breast cancer progression, and amplifications as well as deletions have been described [16–18]. Thus, we selected five other genes and one microRNA for mutational screening, based on functional as well as expression studies and distance to the STR with maximum singlepoint HLOD score (Table 1). Although we found different variants in both coding and non-coding regions, none were deleterious mutations (supplementary table 1).

Since the mutational screening did not reveal any deleterious mutations, we sought to confirm that region rs477138-rs514933 in chromosome 11 was the most likely candidate region. Therefore, we genotyped three additional STRs with high heterozygosity, close to D11S4136, and found that two of them gave high singlepoint LOD scores, which suggests that the region is a strong candidate to carry the disease locus (Fig. 2). The 11q13 region has a high density of genes and our final candidate region of 7 Mb contains around 200 genes. The unknown function of most of the genes in the region may have biased the selection we made, since it was based on known functional features. Further mutational screening within this region is therefore required.

In conclusion, our results suggest that the 11q13 region might carry a breast cancer susceptibility gene. We have narrowed it down from 28 to 7 Mb using both a fine-mapping study and an indirect approach based on HapMap data. We have performed mutational screening in some candidate genes, but have not found any deleterious mutations. A larger study involving additional families linked to this region is required to further narrow down the region and facilitate a more extensive mutational screening to find a causal gene.

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