ORIGINAL ARTICLE

"*BRCA1* 5272-1G>A and *BRCA2* 5374delTATG are founder mutations of high relevance for genetic counselling in Breast/Ovarian cancer families of Spanish origin"

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ABSTRACT

The distribution of *BRCA1* and *BRCA2* germ line mutations in breast/ovarian cancer families varies among different populations, which typically present a wide spectrum of unique mutations. Splicing mutation 5272-1G>A of *BRCA1* and frameshift mutation 5374delTATG of *BRCA2* are highly prevalent mutations in Castilla-León (Spain), accounting for 18.4% and 13.6% of BRCA1 and BRCA2 positive families, respectively. To test the presence of founder effects, nine Spanish 5272-1G>A and thirteen 5374delTATG families were genotyped with polymorphic markers linked to *BRCA1* or *BRCA2*. All the 5272-1G>A families shared a common haplotype in eight markers (1.1 Mb region) and the mutation age was estimated in 15 generations (~380 years). A conserved haplotype associated to 5374delTATG was observed in four markers (0.82 Mb). The mutation occurred approximately 48 generations ago (~1,200 years). Each mutation likely arose from a common ancestor that could be traced to a small area of Castilla-León and expanded to other Spanish regions. They can have significant impact on the clinical management of asymptomatic carriers as well as on the genetic screening strategy to be followed in populations with Spanish ancestries.

Keywords: *BRCA1; BRCA2;* Breast cancer; ovarian cancer; founder mutations

INTRODUCTION

Breast cancer is a multifactorial disease caused by interactions between genetic and non-genetic factors. Among 5-10% of all breast and ovarian cancers are considered hereditary, and about 30-50% of them are due to mutations in the two major susceptibility genes (*BRCA1* and *BRCA2*) (1-3). Approximately 3,500 distinct sequence variants have been described in the BIC database, of which more than 1,700 are pathogenic. Mutations are scattered along the entire coding regions and intronic flanking sequences of both genes. The frequency and incidence of mutations in these genes varies depending on the geographical area or the ethnic group. However, several alterations have been identified in particular populations due to founder effects. To describe a recurrent mutation as a potential founder mutation, it is important to show that the mutation occurred only once in an ancestral individual or common ancestor. To distinguish between single and multiple historical occurrences of the same mutation, it is necessary to genotype DNA variants that are close enough to the mutation to cosegregate together through numerous generations, with little or no separation by meiotic recombination. Likewise, the identification of recombinant polymorphic markers in this study it is essential to estimate the number of generations since the mutation occurred.

Founder mutations have been described in individuals of distinct ancestries (4) including those observed in Ashkenazi Jews (187delAG and 5382insC of *BRCA1* and 6174delT of BRCA2) (5), Iceland (*BRCA2* 995del5) (6), and Portugal (*BRCA2* c.156_157insAlu) (7,8), and other populations (9-11). In the Spanish population mutations, 330A>G of *BRCA1* from Northwestern Spain and 9254delATCAT from the Mediterranean region were described as founder mutations (12,13). The identification of founder mutations contributes to reduce the time and cost of the analysis providing

an efficient design of the mutational screening protocols and genetic counselling in a specific population (14,15).

Since 1998, we have scanned for mutations the *BRCA1* and *BRCA2* genes in low to high risk breast/ovarian cancer patients within the Hereditary Breast and Ovarian Cancer (HBOC) prevention program of Castilla-León (Spain). In previous works we found that 65 out of 459 unrelated families carried a pathogenic mutation (23 *BRCA1* and 42 *BRCA2*) (16-18). We have completed the analysis in another 227 BOC families totalling 686 scanned families, where 104 positive families (15.2%; 38 *BRCA1* and 66 *BRCA2*) were identified. The most frequent mutation of *BRCA1* was 5272-1G>A (c.5153-1G>A according to the Human Genome Variation Society –HGVS- nomenclature) that was found in seven unrelated families (19) while 3036delACAA (HGVS nomenclature c.2808_2811delACAA) and 5374delTATG (HGVS c.5146_5149del4) were the most prevalent of *BRCA2*, that were detected in 12 and 9 families, respectively.

BRCA1 5272-1G>A has been reported only five times in the BIC database or in Spanish families (20,21). This mutation causes an aberrant splicing of exon 19 by deletion of the first nucleotide, which generates a frameshift and a premature stop codon (20,21). The frameshift deletion 5374delTATG truncates the BRCA2 protein at codon 1723 and was described by our group for the first time although it has also been reported by other groups (17,20,22,23). By contrast, the 3036delACAA mutation has been described worldwide in many populations and is the second recurrent pathological mutation in the BIC database ranking. A previous work suggested that it may have multiple different origins (24).

In order to determine the presence of possible founder effects of mutations *BRCA1* 5272-1G>A and *BRCA2* 5374delTATG in Castilla-León we proceeded to study the

haplotypes associated with them in our families and another two 5272-1G>A and four 5374delTATG families that were recruited from other Spanish regions.

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MATERIALS AND METHODS

Patients

Breast and/or ovarian cancer patients from Castilla-León were selected according to the following criteria (16): (I) One breast cancer (BC) ≤ 40 y, (II) two BC, one of them ≤ 50, (III) three or more BC, (IV) BC and ovarian cancer (OC) in the same family or patient, (V) one male breast cancer, (VI) bilateral BC, (VII) two OC, and (VIII) 1 BC + familial aggregation of other types of cancer. A total of 686 families that fulfilled the selection criteria were recruited for BRCA screening. A written informed consent was obtained from all patients.

BRCA1 and *BRCA2* mutational analysis

Genomic DNA was isolated from peripheral blood lymphocytes by standard procedures. The complete coding sequences and the intron-exon boundaries of both genes were analyzed by Conformation Sensitive Gel Electrophoresis (CSGE) and Heteroduplex Analysis by Capillary Array Electrophoresis (HA-CAE) (25-27) (Supplemental Figures 1 and 2), and subsequent DNA sequencing of anomalous patterns using the BigDye Terminator Sequencing Kit v3.1 (Applied Biosystems, Foster City, USA). In order to avoid any confusion, designation of mutations was according to the BIC database (http://research.nhgri.nih.gov/projects/bic/Member/index.shtml); cDNA accession numbers were U14680 (BRCA1) and U43746 (BRCA2). Also, detection of large genomic rearrangements in *BRCA1* was performed in high risk patients (criteria III and IV) with previous negative BRCA test. Multiplex ligation-dependent probe amplification (MLPA) of *BRCA1* (P002-BRCA1; MRC-Holland, Amsterdam, The Netherlands) was carried out according to the manufacturer's instructions.

***BRCA1* 5272-1G>A and *BRCA2* 5374delTATG families.**

A total of 22 HBOC families harbouring either 5272-1G>A or 5374delTATG were recruited from the Genetic Counselling Unit of Hospital General Yagüe (Burgos) and other five Spanish centres (Hospital de Cruces, Barakaldo; Fundación Pública Galega Medicina Xenómica-SERGAS, Santiago de Compostela; Hospital Universitario San Carlos, Madrid; Hospital La Fe, Valencia; and Instituto Catalán de Oncología, Barcelona). Information about the number and type of cancers in each family, age of diagnosis, age of death or current age and geographical origin were collected. DNA samples were obtained from at least one affected member except for one family, where patient sample was not available.

Microsatellite and SNP analysis

Six short tandem repeats (STR) markers spanning a 3.8 Mb interval of chromosome 17 were used for haplotype analysis of 5272-1G>A individuals, two of them were intragenic (D17S855 and D17S1323) and four flanked the *BRCA1* locus (D17S800, D17S1185, D17S1325 and D17S579). Four additional SNPs of the *BRCA1* gene were typed by HA-CAE and subsequent direct sequencing (2731C>T, 3232A>G, 4604-63C>G and 5194-53C>T).

Haplotype analysis for 5374delTATG families was performed using seven STRs (D13S260, D13S1699, D13S1698, D13S171, D13S1767, D13S1695 and D13S1694) flanking the *BRCA2* gene that encompassed a region of 1.34 Mb of chromosome 13 and loci were ordered according to the Ensembl map (www.ensembl.org). SNP 1342C>A of *BRCA2* exon 10 was also genotyped. Primer sequences were obtained from the Ensembl database and standard PCR conditions were used. One primer of each pair was labelled with FAM or HEX fluorochromes. The amplification products were separated on an ABI 3130 Genetic Analyzer and analyzed with the

GeneMapper v3.7 software (Applied Biosystems). STR allele designation was arranged according to Supplemental Tables 1 and 2. These markers were genotyped in index cases and additional family members, and 75 control individuals to estimate allele frequencies.

Haplotype construction and estimation of mutation age.

Haplotype construction was performed manually or with the program PHASE v2.1 (28) based on the genotypes obtained of index cases and relatives and control individuals. Several methods for dating mutations have been developed, all of them based on the calculation of the linkage disequilibrium between the disease allele and linked markers (29,30). The age in generations for both mutations was calculated with the equation $G=\log\delta/\log(1-\theta)$, where δ represents the linkage disequilibrium measure between the mutation and every one of the closest recombinant microsatellite marker and θ is the recombination fraction between a marker and the gene (29).

RESULTS

BRCA1/2 pathological mutations in Castilla-León.

The *BRCA1* and *BRCA2* genes of 686 unrelated families (1067 samples including probands and family members) were scanned for mutations. We identified 104 families carrying a deleterious mutation, 38 *BRCA1* (BRCA1+) and 66 *BRCA2* positive families (BRCA2+), that accounted for 15.2% of positive families in our region. Four recurrent mutations in *BRCA1* and eight in *BRCA2* contributed to the 52.63% and 59.1% of all deleterious mutations of each gene, respectively, that may reflect the presence of region-specific founder effects. The most frequent mutation of *BRCA1* was 5272-1G>A, found in seven BRCA1+ families (18.4%), whereas, 3036delACAA (12 BRCA2+ families; 18.1%) and 5374delTATG (nine families; 13.6%) were the most prevalent alterations of *BRCA2*.

Haplotype generation and age estimation of 5272-1G>A

We typed seven families with the *BRCA1* 5272-1G>A mutation of the east of Castilla-León region and two additional Spanish positive families from the Basque Country and Valencia, one of them with confirmed origin in our region. This mutation is responsible of twelve breast cancer cases (two patients with bilateral BC) and two ovarian cancer cases (both with BC, too). The mean age of diagnosis is 49.9 years ranging from 23 to 76 years for breast cancer and 45.5 years for ovarian cancer (Table 1). A total of 24 family members, 19 of them positive carriers, were available to establish the core haplotype.

We constructed the haplotype with six STR markers and four SNPs (Table 2; Figure 1). The DNA change 5194-53C>T were the most informative SNP as the disease-associated allele is only present in 12% of controls (Table 2). Six of the nine families share the haplotype 6-17-T-G-7-C-T-6-8-11. In three families (117, 178 and

V-4), the disease-associated haplotype showed a recombination in one of the most distal flanking markers, D17S800 or D17S579. This common haplotype was found neither 150 control chromosomes nor non-carrier relatives. Furthermore, the frequencies of STR alleles of the core haplotype were lower than 15% in the control chromosomes, except for the D17S1323 microsatellite where the allele 6 was also the most prevalent in controls (63%).

The linkage disequilibrium measures (δ) between the mutation and each of the recombinant microsatellite markers D17S800 and D17S579 were calculated as δ= 0.7174 and δ= 0.7685, respectively. The recombination fraction (θ) was determined from the physical distance between the mutation and each of the markers, resulting 0.0215 cM for D17S800 and 0.017 cM for D17S579. According to these data the mutation 5272-1G>A arouse approximately between 15.28 and 15.36 generations ago or 382-384 years assuming 25 years per generation.

Haplotype generation and age estimation of 5374delTATG.

In the same way, *BRCA2* 5374delTATG mutation was detected in nine unrelated families from our region. Also, four Spanish families collected from Galicia, the Basque country, Catalonia and Madrid participated in this study. Clinical characteristics of these families are shown in Table 1. Among the carriers there were twenty breast cancer cases (four of them bilateral and one male BC), two OC, four gastric and three prostate cancers among others (Table 1). The average age of female BC cases was 47.1 years (ranging from 28 to 74 years old) and 67 years for OC. A total of 43 family members, 31 of them positive carriers, were used to construct the disease-associated haplotype.

Eight of the thirteen families carried a common haplotype associated to this mutation (9-2-12-2-C-3-6-5-3) (Table 3; Figure 2). This shared haplotype included six

markers covering 0.820 Mb; but D13S1698 or D13S1695 markers recombined in the rest of the families. For these markers the linkage disequilibrium measures obtained were δ= 0.9070 for D13S1698 and δ= 0.74126 for D13S1695. Therefore, the age of the mutation was estimated around 48 generations (46.43-48.85), hence the mutation began to spread between 1160 and 1221 years ago (25 years/generation).

DISCUSSION

Reports of founder mutations in cancer predisposing genes are growing day by day. Their knowledge permits an efficient screening strategy in each population (4,7,31) and they also provide unique opportunities to carry out genotype-phenotype correlations. In the Spanish population there are two proved founder mutations: *BRCA1* 330A>G (Galician origin) (13) and *BRCA2* 9254delATCAT (Mediterranean origin) (12). Both mutations are also frequent in our region, accounting for 13.2% of BRCA1+ families (5/38) and 7.6% (5/66) of BRCA2+ ones, respectively. Herein we provide additional support for another two founder mutations: *BRCA1* 5272-1G>A and *BRCA2* 5374delTATG.

Nucleotide substitution 5272-1G>A is a splicing mutation that abolishes the acceptor site of intron 18 of *BRCA1* but at the same time generates a novel splice site one nucleotide downstream (http://www.fruitfly.org/seq_tools/splice.html), and a premature stop codon and consequently a truncated protein at position 1728 (20). Therefore, it disrupts the essential BRCT domains of the BRCA1 protein (amino acids 1649-1736, 1756-1855). The average age at diagnosis was 49.9 years that is significantly greater than previously reported for mutations in the *BRCA1* gene (~41 years) (32). One possible explanation could be that mutations of the 3'end of *BRCA1* might have a different age-related expressivity, but other mutations of this region do not have a remarkable effect on this parameter, such as 5382insC of Ashkenazi origin with an average onset of 41 years (32). Another alternative explanation could be that the effect on splicing of 5272-1G>A might modify the expressivity of this mutation compared to protein truncating mutations of 3' exons. Again, other splicing mutations of the 3' end have been reported with even lower age at diagnosis than the average of *BRCA1* families (e.g. 5271+1G>C [37.7 years] and 5397-1G>T [38.3

years]) (33). Further studies of 5272-1G>A families are needed to clarify this issue. Among families harbouring this mutation the ratio of breast cancer versus ovarian cancer is 6.5:1, which is in agreement with previous reports (34) that justify a lower proportion of ovarian cancer associated to mutations in the 3' region of the gene.

In East Castilla-León 5272-1G>A mutation was observed in seven unrelated families and is the most prevalent *BRCA1* mutation (18.4%). Intriguingly, this mutation has not been identified in West Castilla-León (35). To date, this mutation has been detected in another three Spanish families outside Castilla-León (Figure 3), one of them could not be included in this study. We confirmed that family BI-1 (Table 1) also came from Burgos, but unfortunately, we could not ascertain the origin of the two remaining families. Additionally, this mutation has been reported in four western European patients at the BIC database. Seven *BRCA1* 5272-1G>A families share an identical haplotype covering a chromosomal region of 3.8 Mb; the remaining two showed recombinations at the most distal markers. Altogether these data indicated a recent origin with a common ancestor 15.3 generations ago or 383 years (25 years per generation). The poor dispersal of this mutation in the rest of Spanish regions might be also attributed to the epidemic diseases that overwhelmed Burgos during the 17th century (http://www.artehistoria.jcyl.es/). The presence of this mutation in Western Europe (BIC database) might be due to the European wars of the Spanish Empire (1516-1714). It would be very interesting to know whether these European families have the same core haplotype. By contrast, one of the most frequent *BRCA1* mutations in Spain and worldwide, 187delAG, is absent in our region (16,21).The explanation may rely on the effective expulsion of non-converted Jews after the Christian "Reconquista" in the 15th century.

With regard to *BRCA2* 5374delTATG, this mutation truncates the BRCA2 protein at codon 1723 disrupting 3 out of the 8 BRC repeats that interact with RAD51, and the entire C-terminal region. The average age at onset of breast cancer was 47.1 years (range 28-74 years) in agreement with previous works that indicated that *BRCA2* is associated with an older age at diagnosis of BC (32,36). This mutation is also associated with prostate cancer in three pedigrees (Table 1) (37). Conversely, another 4-nucleotide deletion only 30 bp upstream (5344delAATA) with a similar effect on the protein (stop codon at 1710) (16) is apparently associated with early onset BC (average 37.7 years, range 29-55), although the number of cases (6 BC) is not significant.

Since it was reported for the first time by our group in two unrelated families (17), we have identified seven more pedigrees, which account for 13.6% of BRCA2+ families (Figure 3). Afterwards, it was found in other regions of Northern Spain (20,22) or in Hispanic Countries (38). All the collected families shared an ancestral haplotype enclosing a 0.82 Mb region. Family 672 showed a recombination event between D13S260 and D13S1699. We estimated that this DNA change was originated 48 generations ago (about 1190 years), when the Iberian territory remained under Islamic dominion, and began the conquest and a repopulation process of the region by the Northern Christian kingdoms (http://www.artehistoria.jcyl.es/histesp/lugares/272.htm). By this time, Burgos was part of the Southern border of the Asturias Kingdom with the Muslim Emirate of Cordoba. Thus, it would be also very interesting to know the prevalence of 5374delTATG in Southern Spain or even in the Maghreb countries, where it might hypothetically represent a relevant genetic risk factor although until now full data from

these regions have not been published. Its identification in two Chilean families also supports the hypothesis that it expanded before the colonization in 1492 (38).

Both mutations, 5272-1G>A and 5374delTATG, account for around 1 in 6 of deleterious mutations of both genes in families from Castilla-León (15.4% of BRCA1/2 positive families). Direct screening of these mutations could be undertaken as a first step for molecular analysis allowing for a more rapid, accurate and less expensive test. In our population, both mutations are initially tested by HA-CAE of the corresponding exons (26) that provides clear distinguishable patterns for each mutation that is subsequently confirmed by sequencing (see Materials and Methods and Supplemental Figures 1 and 2). Moreover, precise estimates of the prior probability of a mutation carrier developing cancer should be possible (39).The final goal of the genetic testing is to focus on prevention strategies to reduce the risk of cancer in asymptomatic carriers. The oncologist recommends annual determination of CA-125 (cancer antigen 125); gynecologic and mammographic and/or breast magnetic resonance surveillance, transvaginal ultrasonography and lastly, prophylactic surgery (bilateral mastectomy, salpingo-oophorectomy) to the *BRCA1* 5272-1G>A and *BRCA2* 5374delTATG asymptomatic carrier women. Recommendations for the *BRCA2* 5374delTATG male carriers included an annual breast examination and prostate screening (PSA –Prostate Specific Antigen- and digital rectal examination).

Previous works have indicated that mutational events are not random and occur at physiologically stable DNA secondary structures called hairpin-loops (40,41). Interestingly, 5374delTATG is placed in the single-stranded portion of a hairpin-loop structure that is more vulnerable to spontaneous mutagenesis (Supplemental Figure 3) (40,41). Mutation 5272-1G>A is located in double-hairpin structure where the

nucleotide 5272-1 is apparently a hotspot with two different changes: G to A and G to C. Furthermore, another important BIC mutations surrounding both, *BRCA2* 5298A>C and 5301insA (39 and 29 BIC entries, respectively), and *BRCA1* 5277A>G (15 BIC entries) are located in these “mutagenic” structures. These data suggest that most important mutations of the BRCA genes could have been generated by this mechanism.

In conclusion, each of these mutations likely arose from a common ancestor that could be traced to a small area of the provinces of Burgos and Palencia (539.253 inhabitants; shadowed in Figure 3). Both mutations are responsible for 27.6% of the 58 BRCA+ families from this specific area and the 5272-1G>A mutation alone is carried by 39% of BRCA1+ families (7/18). Moreover, the constant migratory movements of the Castilla-León population (http://www.ine.es/inebmenu/mnu_cifraspob.htm) might also have spread these mutations to Central-Southern Spain and even Latin American countries where they might represent important cancer risk alleles. Finally, founder mutations are useful to design an efficient mutational screening in a specific population, and estimating the age of the more recent common ancestor may improve our understanding of the population genetics of HBOC. The identification of founder effects will also facilitate the discovery of other genes and/or lifestyle modifiers that could elucidate the phenotypic heterogeneity as well as differences in penetrance and expression in families carrying the same mutation.

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FIGURE LEGENDS

Figure 1. Segregation of the haplotype linked to mutation 5272-1G>A in family CYL117. Ancestral haplotype is boxed; the index case is indicated by an arrow; black circles indicate disease positive relatives and asymptomatic carriers are shown as half-filled circles. Some microsatellites were not analyzed (n.a.) because no more DNA sample was available.

Figure 2. Segregation of the haplotype linked to mutation 5374delTATG in family CYL239. Ancestral haplotype is boxed; the index case is indicated by an arrow; black circles indicate disease positive relatives and an asymptomatic carrier male is shown as a half-filled square. The asterisk indicates a phenocopy not associated with this mutation. Some microsatellites were not analyzed (n.a.) because no more DNA sample was available.

Figure 3. Geographic distributions of the families carriers of the two founder mutations in Spain (left) and Castilla-León (right). 5272-1G>A families are represented with black boxes and 5374del4 are shown as black circles. Most families are concentrated in a small area comprising part of the provinces of Burgos and Palencia that are shadowed (right).

Table 1. Clinical characteristics of families.

Family	Proband		Cancer cases in relatives (age at onset) [b]
	Age [a]	Selection criteria	
5272-1G>A			
117	49	III	2 BC (**66, 76**); 1 CRC (**60**)
175	47^{OC}-51	III	3 BC (29, others >50); 1 esophagus (62); 1 tongue (53)
178	27-47^{bi}	VI	2 OC (57, 57^{c}); 1 CRC + leukemia (81+83^{c}); 1 Gastric (64)
302	44	III	1 BC (46^{c}); 1 OC (47^{c})
359	42-44^{OC}	III	2 BC (45^{c}, 46); 1 gastric (82); 1 hepatic (47^{c})
609	54	VIII	1 BC (80^{c}); 1 esophagus (61^{c}); 1 bladder (61^{c})
663	46-46^{bi}	II	1 BC (**51**)
BI-1	23	III	2 BC (53^{c},64^{c}.); 1 lung (65^{c}); 1 gastric (68^{c})
V-4	48	II	1 BC+OC (59^{BC}+65^{OCc});1 lung; 1 gastric
5374delTATG			
126	68	III	3 BC (43^{c}, **66**, 87^{c}), 2 lung (55^{c}, **68**)
135	29	III	2 BC (**34**, **47**)
239	28	III	3 BC (**31**^{c}, 58, **50**)
287	50	III	2 BC (42, 42^{c}); 1MBC (**71**^{PR}**+78**^{BC}); 1 PR (60^{c})
353	0 (59)	II	2 BC (32^{c}, 58^{c})
534	40	II	1 BC (**59**)
625	58-66^{bi,c}	II	1 BC (40^{c}), 1 gastric (70^{c})
672	44^{c}	III	3 BC (42^{bi}, 49^{c}, 83), 2 OC (35, 63)
674	67^{OC}	III	2 BC (**58**, 63^{c}), 1 OC (56^{c})
Gal	28-38^{bi}	II	1 BC (42^{c}); 2 gastric (60^{c}, 66^{c}); 1 PR (70^{c})
M-179	49	II	1 BC (**74-75**^{bi}); larynx (37^{c}); gastric (45^{c}); hepatic (65^{c}); abdominal (64^{c})
B-6	40-44^{bi}	III	2 BC (39, 56); 1 bladder (85^{c}), 1 lung
BI-3	67^{OC}	III	5 BC (28^{c}, 42, **44**, 46^{c}, 52); 1MBC (47^{BC}+50^{PR,c}); 1 PR; 2 CRC (50^{c}, 75^{c}); larynx (47^{c})

Types of cancer: BC, breast cancer; OC, ovarian cancer; bi, bilateral; MBC, male breast cancer; PR, prostate cancer, CRC; colorectal cancer.

[a] In family 353 the proband is healthy; within brackets is the current age. Probands of families 625 and 672 died at the age of 68 and 52, respectively.

[b] Carrier relatives are shown in bold type.

[c] Deceased patients.

Table 2. Haplotype analysis of *BRCA1* 5272-1G>A families.

Marker	Position (Mb)	FAMILY *									CONTROLS (75)
		117 (8)	175 (1)	178 (3)	302 (1)	359 (4)	609 (1)	663 (2)	BI-1 (2)	V-4 (2)	
D17S800	2.150	**6**	3/**6**	5	**6**/6	**6**	5/**6**	**6**	**6**	5	**6** (13%)
D17S1185	0.655	**17**	13/**17**	**17**	15/**17**	**17**	11/**17**	**17**	**17**	**17**	**17** (9%)
2731C>T	exon 11	**T**	C/**T**	**T**	C/**T**	**T**	T/**T**	**T**	**T**	**T**	**T** (48.3%)
3232A>G	exon 11	**G**	A/**G**	**G**	A/**G**	**G**	G/**G**	**G**	**G**	**G**	**G** (48.3%)
D17S855	intron 12	**7**	2/**7**	**7**	6/**7**	**7**	**7**/8	**7**	**7**	**7**	**7** (10%)
4604-63C>G	intron 14	**C**	C/**C**	**C**	C/**C**	**C**	G/**C**	**C**	**C**	**C**	**C** (51%)
5194-53C>T	intron 17	**T**	C/**T**	**T**	C/**T**	**T**	C/**T**	**T**	**T**	**T**	**T** (12%)
BRCA1	5272-1G>A										
D17S1323	intron 20	**6**	**6**/**6**	**6**	**6**/**6**	**6**	3/**6**	**6**	**6**	**6**	**6** (63%)
D17S1325	0.430	**8**	**8**/14	**8**	**8**/13	**8**	**8**/12	**8**	**8**	**8**	**8** (2%)
D17S579	1.700	7	9/**11**	**11**	**11**/12	**11**	3/**11**	**11**	**11**	10	**11** (2%)

* The number of relatives tested is between brackets. Shared alleles are in bold type. The allele's frequencies were calculated from 75 controls.

Table 3. Haplotype analysis of 5374delTATG-BRCA2 families.

Marker	Position (Mb)	Castilla-León (CYL) FAMILIES*									NON-CYL FAMILIES*				CONTROLS (75)
		126 (8)	135 (5)	239 (6)	287 (4)	353 (1)	534 (4)	625 (4)	672 (3)	674 (2)	GAL (3)	M179 (1)	B6 (1)	BI-3 (4)	
D13S260	-0.480	8	**9**	**9**	**9**	8/**9**	**9**	**9**	6	**9**	**9**	8/**9**	8/**9**	11	**9** (18%)
D13S1699	-0.310	**2**	**2**	**2**	**2**	**2**/2	**2**	**2**	**2**	**2**	**2**	**2**/2	**2**/3	**2**	**2** (56%)
D13S1698	-0.210	**12**	**12**	**12**	**12**	3/**12**	**12**	**12**	4	**12**	**12**	10/**12**	**12**/11	**12**	**12** (23%)
D13S1697	-0.050	**2**	**2**	**2**	**2**	**2**/2	**2**	**2**	**2**	**2**	**2**	**2**	**2**/3	**2**	**2** (78%)
1342C>A	exon 10	**C**	**C**	**C**	**C**	**C**/A	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C** (28%)
BRCA2	5374delTATG														
D13S1701	0.230	**3**	**3**	**3**	**3**	**3**/4	**3**	**3**	**3**	**3**	**3**	2/**3**	**3**/6	**3**	**3** (27%)
D13S171	0.340	**6**	**6**	**6**	**6**	**6**/8	**6**	**6**	**6**	**6**	**6**	**6**/8	**6**/7	**6**	**6** (41%)
D13S1695	0.610	**5**	**5**	**5**	**5**	**5**/11	**5**	**5**	**5**	**5**	**5**	6/7	6/6	6	**5** (6%)
D13S1694	0.860	**3**	**3**	**3**	**3**	**3**/3	**3**	**3**	**3**	**3**	**3**	**3**/3	**3**/4	**3**	**3** (43%)

*The number of relatives tested is between brackets. Shared alleles are in bold type. The allele's frequencies were calculated from 75 controls.

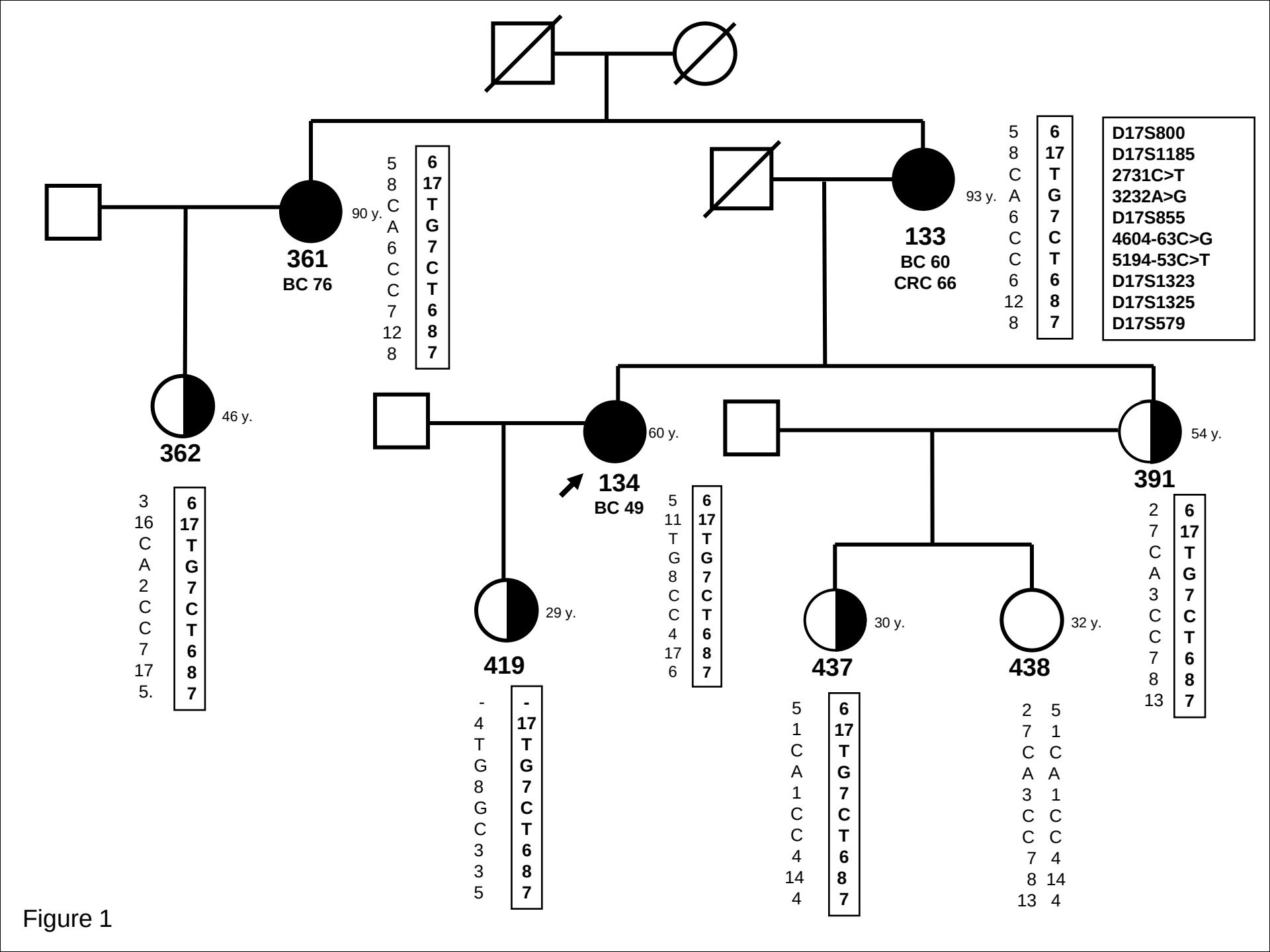

90 y.
361
BC 76
5 8 C A 6 C C 7 12 8
6 17 T G 7 C T 6 8 7
93 y.
133
BC 60
CRC 66
5 8 C A 6 C C 6 12 8
6 17 T G 7 C T 6 8 7
D17S800
D17S1185
2731C>T
3232A>G
D17S855
4604-63C>G
5194-53C>T
D17S1323
D17S1325
D17S579
46 y.
362
3 16 C A 2 C C 7 17 5.
6 17 T G 7 C T 6 8 7
60 y.
134
BC 49
5 11 T G 8 C C 4 17 6
6 17 T G 7 C T 6 8 7
54 y.
391
2 7 C A 3 C C 7 8 13
6 17 T G 7 C T 6 8 7
29 y.
419
- 4 T G 8 G C 3 3 5
- 17 T G 7 C T 6 8 7
30 y.
437
5 1 C A 1 C C 4 14 4
6 17 T G 7 C T 6 8 7
32 y.
438
2 7 C A 3 C C 7 8 13
5 1 C A 1 C C 4 14 4

Figure 1

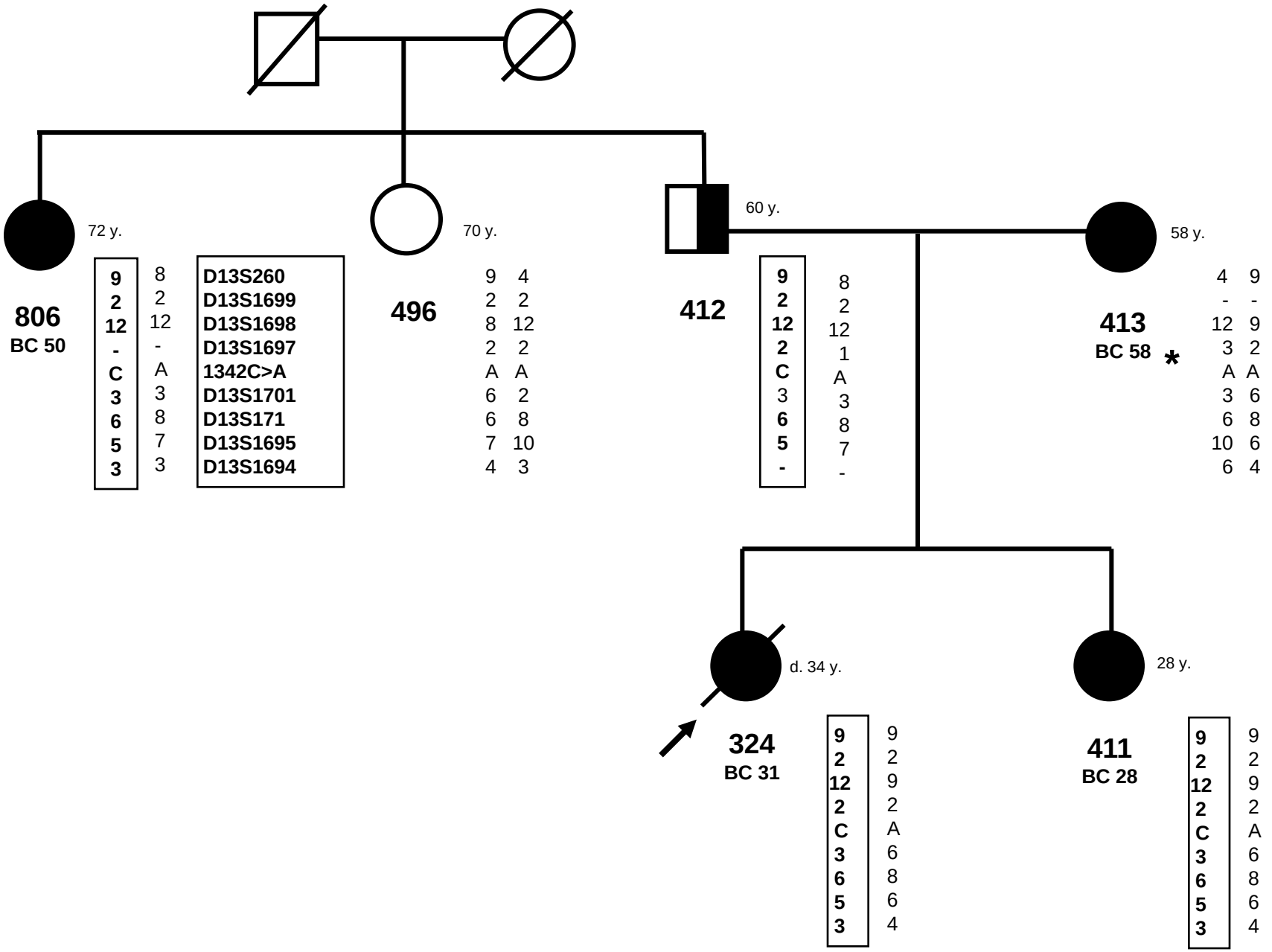

72 y.
806
BC 50
9 8
2 2
12 12
- -
C A
3 3
6 8
5 7
3 3
D13S260
D13S1699
D13S1698
D13S1697
1342C>A
D13S1701
D13S171
D13S1695
D13S1694
70 y.
496
9 4
2 2
8 12
2 2
A A
6 2
6 8
7 10
4 3
60 y.
412
9 8
2 2
12 12
2 1
C A
3 3
6 8
5 7
- -
58 y.
413
BC 58
*
4 9
- -
12 9
3 2
A A
3 6
6 8
10 6
6 4
d. 34 y.
324
BC 31
9 9
2 2
12 9
2 2
C A
3 6
6 8
5 6
3 4
28 y.
411
BC 28
9 9
2 2
12 9
2 2
C A
3 6
6 8
5 6
3 4

Figure 2

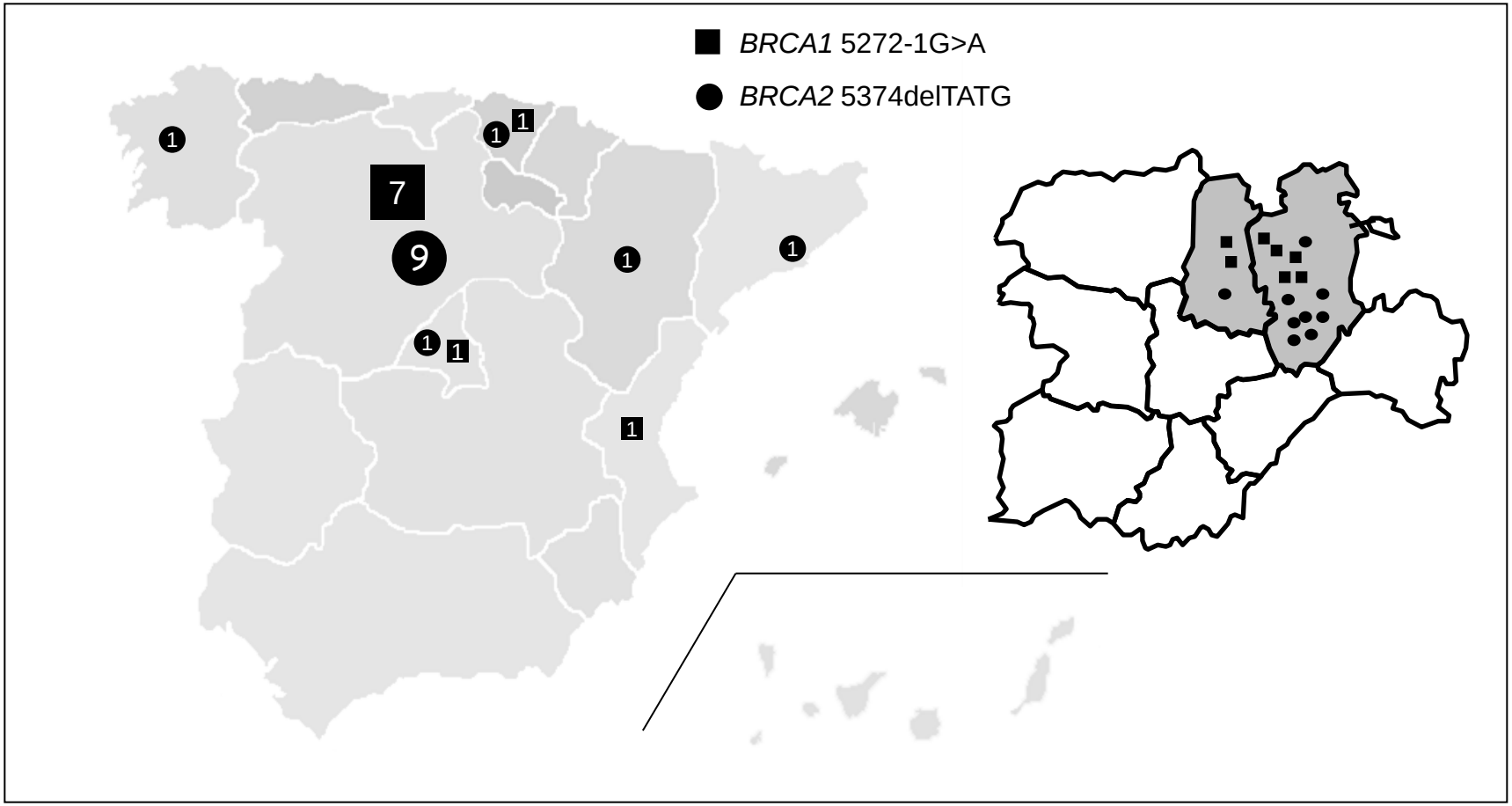
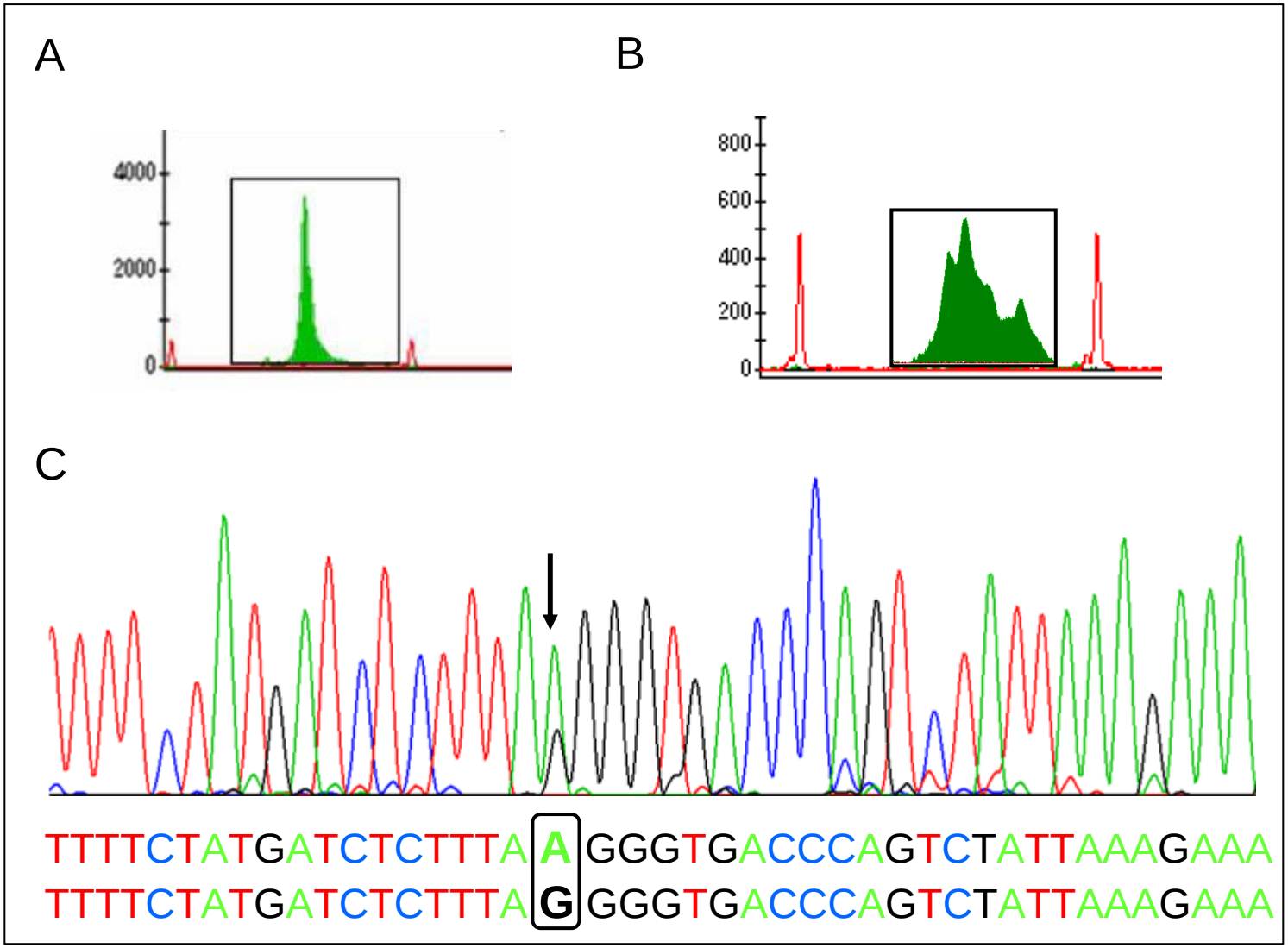
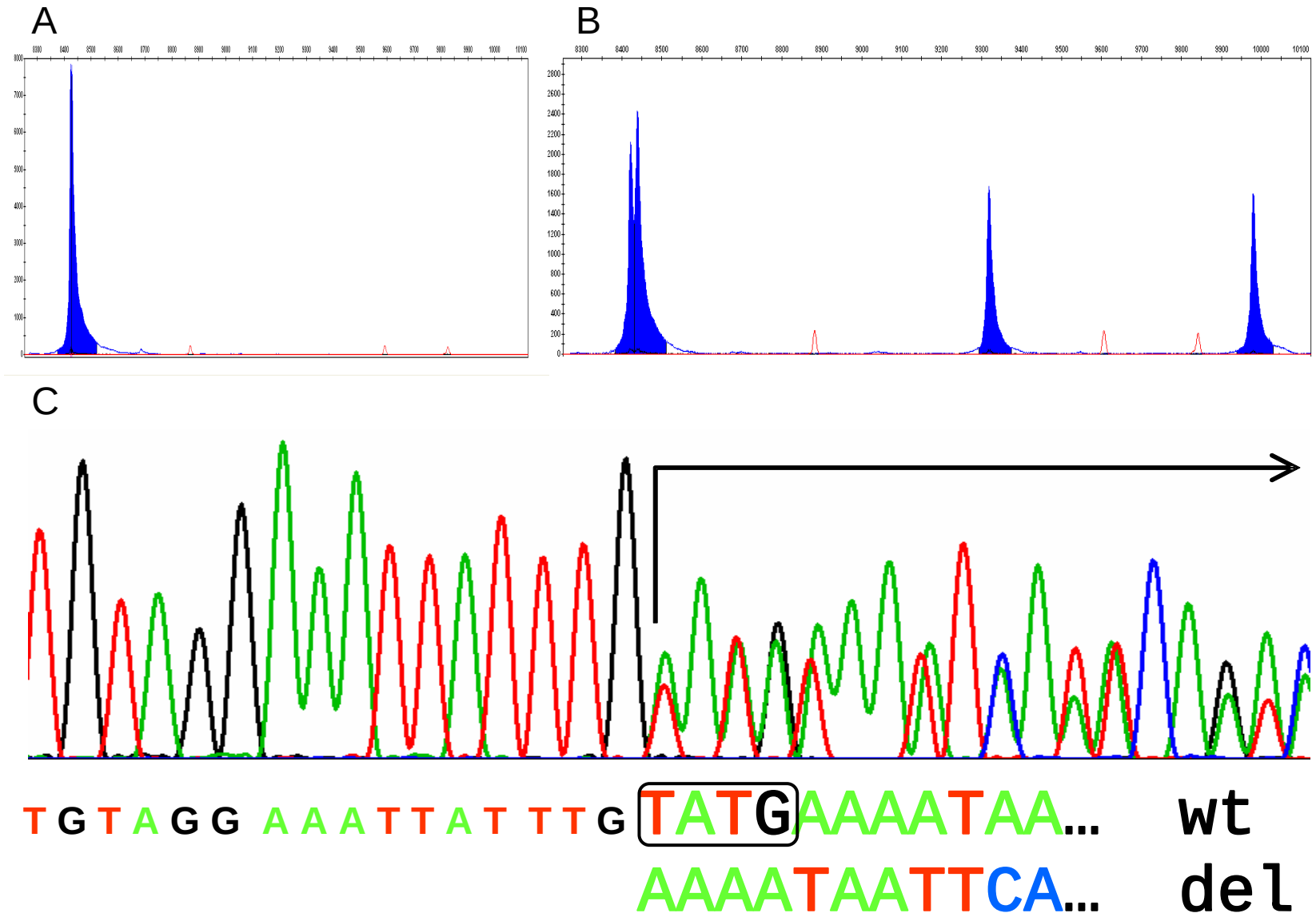


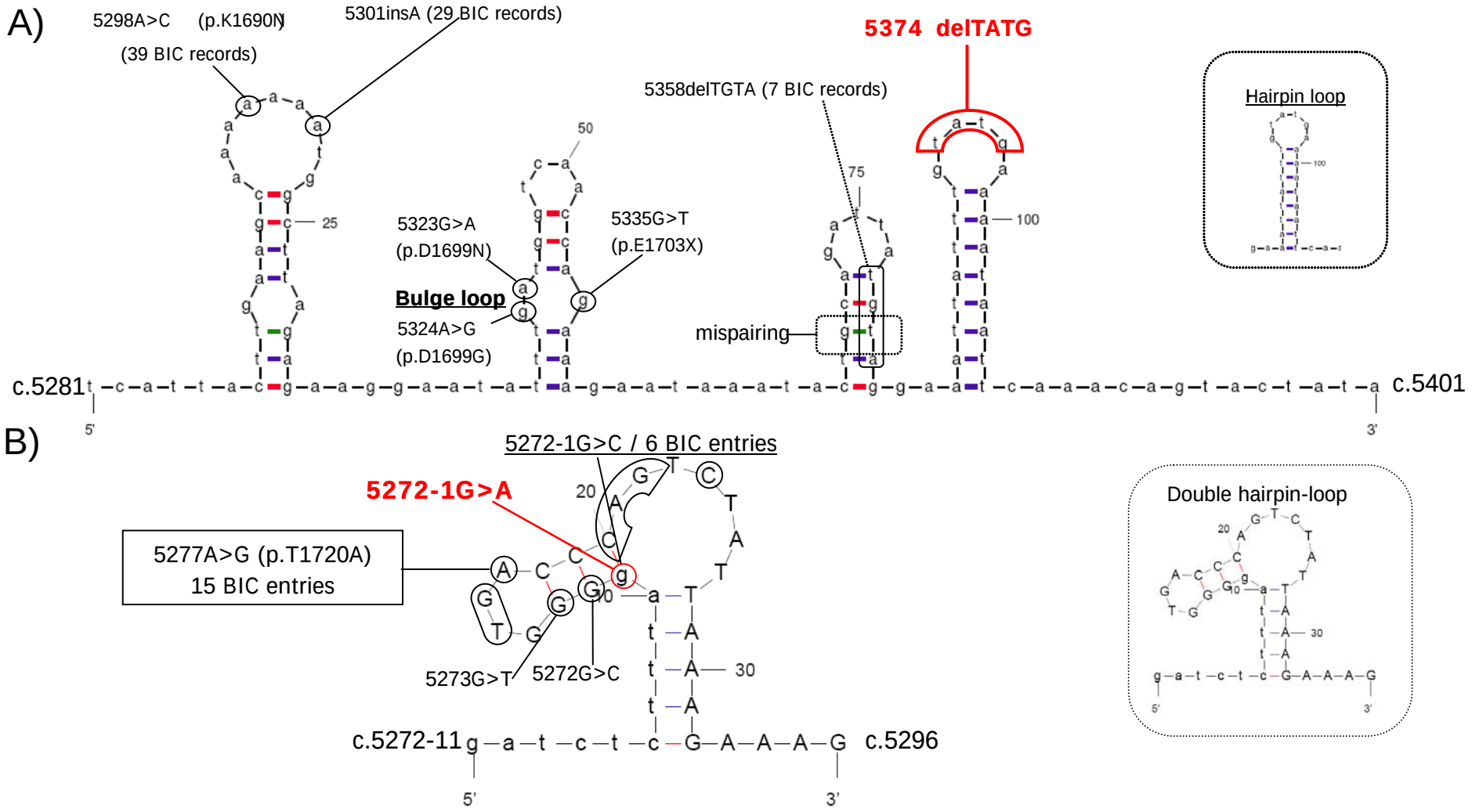
Figure 3

Supplementary Figure S1. GeneMapper screenshots of BRCA1-exon 19 detected by HA-CAE. Scan points are in x-axe and relative fluorescent units (RFU) are in y-axe. (A) Normal pattern. (B) 5271-1G>A pattern. (C) Sequence chromatogram of exon 19 where the nucleotide substitution is shown.



Supplementary Figure S2. GeneMapper screenshots of BRCA2-fragment 11R detected by HA-CAE. Scan points are in x-axe and relative fluorescent units (RFU) are in y-axe. (A) Normal pattern. (B) 5374delTATG pattern. (C) Sequence chromatogram of Fragment 11R of exon 11 where the deletion is boxed.





Supplementary Figure S3. DNA secondary structure of regions surrounding mutations 5374delTATG (A) and 5272-1G>A (B) obtained with the program Quikfold (http://dinamelt.bioinfo.rpi.edu/quikfold.php; Markham & Zuker, 2008). Although other secondary structures are possible, only the most stable thermodynamic structures are shown. A) Nucleotides of founder mutation 5374delTATG (shown in red) are placed in a hairpin loop. Other important mutations from the BIC database are indicated. The most prevalent mutations of this region are placed in another hairpin loop (5298A>C and 5301insA) or anomalous structures with mispaired nucleotides (e.g. 5358delTGTA). Another mutations placed in bulge loops are shown in the figure. B) Double hairpin-loop structure of DNA segment surrounding mutation 5272-1G>A (shown in red). Upper case, exonic sequence; lower case, intronic sequence. This nucleotide is also affected by another mutation (5272-1G>C). Another six BIC mutations of this DNA segment are shown. Origin of all of these mutations seems to be correlated with their presence in specific DNA secondary structures (single-stranded DNA, bulge loops or, specially,hairpin-loops) that are particularly vulnerable to mutagens.

Supplementary table S1. Microsatellite markers linked to the BRCA1 gene that have been typed in CyL population in this study. Allele frequencies were calculated in 150 control chromosomes.

D17S800			D17S1185			D17S855			D17S1323			D17S1325			D17S579		
Frequency	Size	Allele	Frequency	Size	Allele	Frequency	Size	Allele	Frequency	Size	Allele	Frequency	Size	Allele	Frequency	Size	Allele
0.07	165	7	0.03	197	18	0	133	12	0.03	147	8	0.01	180	20	0.12	111	13
0.13	*167*	*6*	*0.09*	*201*	*17*	0.01	141	11	0.07	149	7	0.01	184	19	0.09	113	12
0.53	**169**	**5**	0.16	205	16	0.01	143	10	***0.63***	***151***	***6***	0.01	186	18	*0.02*	*115*	*11*
0.04	171	4	0	207	14	0.07	145	9	0.03	153	5	0.04	190	17	0.02	117	10
0.16	173	3	0.11	209	15	0.20	147	8	0.07	155	4	0.05	192	16	0.04	119	9
0.06	175	2	0.18	213	13	*0.10*	*149*	*7*	0.14	157	3	0.02	194	15	0.09	121	8
0.01	177	1	0	215	12	0.18	151	6	0.03	159	2	**0.30**	**196**	**14**	**0.22**	**123**	**7**
			0.22	**217**	**11**	**0.22**	**153**	**5**	0	161	1	0.18	198	13	**0.22**	**125**	**6**
			0.02	219	10	0.15	155	4				0.10	200	12	0.11	127	5
			0.12	221	9	0.05	157	3				0.05	202	11	0.02	129	4
			0.02	223	8	0.01	159	2				0.07	204	10	0.02	131	3
			0.03	225	7	0	161	1				0.03	206	9	0.02	133	2
			0.01	227	6							*0.02*	*208*	*8*	0.01	135	1
			0.01	231	5							0.01	210	7			
			0	235	4							0.01	212	6			
			0	237	3							0.01	214	5			
			0.03	239	2							0.05	216	4			
			0	243	1							0.01	218	3			
												0.01	222	2			
												0.01	228	1			

The size of alleles is in bp. The most frequent allele in controls is in bold type whereas the alleles segregating with the disease in 5272-1G>A families are in italics. Alleles that show frequency 0 are present in carrier families but not in the control samples.

Supplementary table S2. Microsatellite markers linked to the BRCA2 gene that have been typed in CyL population in this study. Allele frequencies were calculated in 150 control chromosomes.

D13S260			D13S1699			D13S1698			D13S1697		
Frequency	Size	Allele	Frequency	Size	Allele	Frequency	Size	Allele	Frequency	Size	Allele
0	143	13	0.01	140	4	0	153	15	0	217	4
0	149	12	0.38	144	3	0	155	14	0.19	221	3
0.09	151	11	***0.56***	***148***	***2***	0.08	157	13	***0.78***	***225***	***2***
0.03	153	10	0.05	152	1	*0.23*	*159*	*12*	0.03	229	1
0.18	*155*	*9*				0.08	161	11			
0.28	**157**	**8**				**0.27**	**163**	**10**			
0.05	159	7				0.03	165	9			
0.16	161	6				0.03	167	8			
0.16	163	5				0.05	169	7			
0.02	165	4				0.01	171	6			
0.02	167	3				0.04	173	5			
0.01	169	2				0.10	175	4			
0	171	1				0.06	177	3			
						0.01	179	2			
						0.01	183	1			

D13S1701			D13S171			D13S1695			D13S1694		
Frequency	Size	Allele	Frequency	Size	Allele	Frequency	Size	Allele	Frequency	Size	Allele
0.01	280	8	0.09	191	8	0.03	237	12	0.01	224	7
0.02	284	7	0.34	193	7	0.02	241	11	0.26	228	6
0.13	288	6	***0.41***	***195***	***6***	0.08	243	10	0.04	230	5
0.21	292	5	0.01	197	5	0.01	247	9	0.12	232	4
0.28	**296**	**4**	0	201	4	0.01	249	8	***0.43***	***234***	***3***
0.27	*300*	*3*	0.01	203	3	**0.40**	**251**	**7**	0.13	236	2
0.07	304	2	0.14	205	2	0.30	253	6	0.01	238	1
0.01	308	1	0	207	1	*0.06*	*255*	*5*			
						0.03	257	4			
						0.03	259	3			
						0.02	261	2			
						0.01	263	1			

The size of alleles is in bp. The most frequent allele in controls is in bold type whereas the alleles segregating with the disease in 5374_5377delTATG families are in italics. Alleles that show frequency 0 are present in carrier families but not in the control samples.