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Original Article

DNA repair genes implicated in triple negative familial non-BRCA1/2 breast cancer predisposition

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Abstract: Among breast cancers, 10 to 15% of cases would be due to hereditary risk. In these familial cases, mutations in *BRCA1* and *BRCA2* are found in only 15% to 20%, meaning that new susceptibility genes remain to be found. Triple-negative breast cancers represent 15% of all breast cancers, and are generally aggressive tumours without targeted therapies available. Our hypothesis is that some patients with triple negative breast cancer could share a genetic susceptibility different from other types of breast cancers. We screened 36 candidate genes, using pyrosequencing, in all the 50 triple negative breast cancer patients with familial history of cancer but no *BRCA1* or *BRCA2* mutation of a population of 3000 families who had consulted for a familial breast cancer between 2005 and 2013. Any mutations were also sequenced in available relatives of cases. Protein expression and loss of heterozygosity were explored in tumours. Seven deleterious mutations in 6 different genes (*RAD51D, MRE11A, CHEK2, MLH1, MSH6, PALB2*) were observed in one patient each, except the *RAD51D* mutation found in two cases. Loss of heterozygosity in the tumour was found for 2 of the 7 mutations. Protein expression was absent in tumour tissue for 5 mutations. Taking into consideration a specific subtype of tumour has revealed susceptibility genes, most of them in the homologous recombination DNA repair pathway. This may provide new possibilities for targeted therapies, along with better screening and care of patients.

Keywords: Triple-negative breast cancer, familial non-*BRCA1/2* breast cancer, genetic susceptibility, candidate genes, pyrosequencing, DNA repair genes

Introduction

Among all breast cancers, one in ten is associated with hereditary risk. However, mutations in the two major predisposing genes, *BRCA1* [MIM: 113705] and *BRCA2* [MIM: 600185], are found in only 15% to 20% of hereditary breast cancer families [1]. Other genes have emerged, such as *TP53* [MIM: 191170], *PTEN* [MIM: 601728], *STK11* [MIM: 602216] or *CDH1* [MIM: 192090] that are known to cause familial syndromes in which breast cancer (BC) incidence is highly increased, or *BRIP1* [MIM: 605882], *ATM* [MIM: 607585], *CHEK2* [MIM: 604373] or *PALB2* [MIM: 610355] [2], that are associated with a moderate increased risk of breast cancer. But they together represent only about 20% of familial breast cancers. For the vast remaining majority, no mutation is found.

Moreover, breast cancer represents a complex and heterogeneous disease, with different clinical features, response to treatment, histopathology and gene expression profiles. These elements overlap and several large groups of breast with biological and clinical traits in common have been proposed. Because the majority of studies of familial breast cancer grouped all subtypes together, it has been difficult to identify new causal genes, if the causes of different subtypes have different genetic origins. It may thus be pertinent to evaluate genetic predisposition to a specific subtype of breast cancer.

Five types of breast cancer have been identified by gene expression profile [3, 4]; two luminal receptor positive subgroups (A and B), a human epidermal growth factor 2 (HER2) overexpress-

Table 1. Characteristics of the studied population

Characteristics	Patients, n=50	Controles, n=51
Mean age at breast cancer diagnostic (patients) or at sample collection (controls)	48.6	49.6
Age at diagnosis (or collection for controls)		
≤40 years	15	14
between 41 and 50 years	11	10
>50 years	24	27
Family history of cancer		
Family with breast cancer only	4	na
Breast and ovarian family	5	na
Breast and other cancers	46	na
Breast cancer family history		
1 breast cancer ≤35 years	4	na
2 breast cancers	19	na
≥3 breast cancers	27	na

na*: not applicable.*

ing group, a normal breast-like or unclassified group, and a basal-like group that is largely triple-negative breast cancer (TNBC). Later, a claudin-low group was also described (consisting mainly of TNBC).

Triple-negative breast cancers, defined by no expression of estrogen receptor (ER), progesterone receptor (PR), or human epidermal growth factor receptor 2 (HER2) in tumour material, stand for 10-20% of all breast cancers. TNBC are aggressive tumours, affect frequently young patients and have poor clinical outcome. Treatment of TNBC has been challenging due to the absence of well-defined molecular targets.

Genetic predisposition to triple-negative breast cancers has been little studied, except for the two main genes *BRCA1* and *BRCA2* mutations. For TNBC, studies have shown that 9-14% overall and ~20% of cases diagnosed under age 50 harbour germline *BRCA1* mutations [5]. This observation opened new potential therapeutic approaches, such as the anti-PARP1. Mutations in *BRCA2* are associated to a lesser extent with TNBC, with 3.9% of triple-negative tumours patients having a germline mutation in this gene [6]. Few other breast cancer susceptibility genes have been evaluated for mutations in TNBC [7, 8].

Our hypothesis is that some patients with familial TNBC could share a genetic susceptibility to cancer different from other types of breast cancer. To explore this idea, we analysed thirty-six candidate genes known or suspected to be part of the DNA repair process, potentially involved in breast cancer genesis in general or in TNBC specifically.

Materials and methods

Patients

Among 3000+ families screened for a familial breast cancer between 2005 and 2013 at the oncogenetics consultation of the Centre Jean Perrin (France), we collected the samples of all the triple negative breast cancer patients with familial history of cancer but no *BRCA1* or *BRCA2* mutation. In this population, a familial context of cancer is defined as a first degree relative affected with breast cancer or a second degree relative if related by a man, or belonging to a family with a Manchester score >12 (score weighted on anatomopathology) [9]. We included as cases all the patients with a triple negative breast cancer, tested negative for mutations and large genomic rearrangements in *BRCA1* and *BRCA2*, which represent 50 TNBC patients. The control group was composed of 51 samples of DNA from women free of neoplasia [10], matched on age with cases. With this population size and a one-sided alpha =0.05, we reached a power of 70% (Poisson's law of small numbers [11]).

The mean age at breast cancer diagnosis was 48.6 years old (**Table 1**). Genomic DNA from

these patients, their clinical and pathological information, genealogical trees and tissue samples were provided by the oncogenetic department and the anatomopathology department, at the Centre Jean Perrin (Clermont-Ferrand, France).

Each mutation discovered in a proband was sought in all her relatives with available DNA.

All subjects signed an informed consent sheet that was approved by the Regional Ethics Committee (Auvergne, France).

Enrichment

We used the solution-based NimbleGen® Sequence Capture EZ Choice (Roche®) to enrich genomic DNA in the coding sequence of the 36 candidate genes. For each gene, all exons and intron-exon boundaries were covered by the capture.

These genes were known or suspected to be part of the DNA repair process (*POLB* [MIM: 174760], *ERCC1* [MIM: 126380], *APC* [MIM: 611731], *EPCAM* [MIM: 185535], *MLH1* [MIM: 120436], *MLH3* [MIM: 604395], *MSH2* [MIM: 609309], *MSH6* [MIM: 600678], *MUTYH* [MIM: 604933], *PMS1* [MIM: 600258], *PMS2* [MIM: 600259], *PTEN*, *TP53*, *ATM*, *AURKA* [MIM: 603072], *CHEK2*, *CDH1*, *NRIP1* [MIM: 602490], *FGFR2* [MIM: 176943], *STK11*, *WRN* [MIM: 604611]), or to be actor in the BRCA1 pathway (*BRIP1*, *BARD1* [MIM: 601593], *COBRA1* [MIM: 611180], *BRAP* [MIM: 604986], *BRCC3* [MIM: 300617], *PALB2* [MIM: 610355], *BAP1* [MIM: 603089], *BLM* [MIM: 604610], *MRE11A* [MIM: 600814], *NBN* [MIM: 602667], *RAD50* [MIM: 604040], *RAD51D* [MIM: 602948], *RAD51C* [MIM: 602774], *RAD51D* [MIM: 602954]), the most implicated in TNBC so far. We also studied one gene, *BABAM1* [MIM: 612766], located at locus 19p13.1 identified in genome wide association studies to be associated specifically with TNBC (and not with breast cancer in general) [12].

Sample preparation and next generation sequencing

DNA was extracted from peripheral blood with Macherey Nucleo Spin blood X kit, before storage in a DNA Bank.

We used the Roche® Rapid Library Preparation method for sequencing on GS-FLX 454 Roche®. DNA was fragmented by nebulization, ligated to Roche 454 adaptators and purified with AmpureXP beads. The quality of the library was assessed with a Bioanalyzer 2100 (Agilent) and an Infinite 200 plate reader (Tecan). Libraries were then amplified by ligation-mediated PCR (LM-PCR), in which three indexed samples were combined equimolarly. The enrichment process was done with the NimbleGen SeqCap EZ Library LR method, with a double capture. Em-PCR targeting 2 copies per beads was performed prior to sequencing on a GS-FLX 454 Roche®, with Titanium kits.

Bioinformatic analysis

GS Reference Mapper v2.8 (Roche) was used to ensure demultiplexing of the samples, read alignment against the reference genome (hg19 of March 2012) and variant calling. The output files were then filtered by homemade software to sort variants according to minimum coverage (>5 reads) and frequency (>20%). Variant annotation was performed using Alamut Batch software v1.1.2 (Interactive Biosoftware, Rouen, France).

Non-synonymous missense substitutions: Population allele frequencies were extracted from the Exome Variant Server (http://evs.gs.washington.edu/EVS), 1000 Genomes (http://www.1000genomes.org), and dbSNP (version 137; http://www.ncbi.nlm.nih.gov/projects/SNP). Predicted deleterious missense mutations were selected using algorithms (SIFT, MAPP and AlignGVGD) [13] (http://agvgd.iarc.fr). When two algorithms out of three predicted a variation to be deleterious, it was retained for further analysis. We also confirmed prediction for retained variants with PolyPhen2 [14].

Insertion or deletion: The Roche 454 pyrosequencing technology is known to generate numerous homopolymer-associated single base errors [15], which create artifactual insertions or deletions. All in/del variations found within a homopolymer were filtered out with a spectrum based error corrector for 454 sequencing data, named HECTOR [16].

Splicing variants: The potential impact of variants located on splicing junctions was evaluated using SpliceSiteFinder, MaxEntScan, and GeneSplicer prediction software [17, 18]. Variants predicted to alter splicing by at least

Table 2. Mutations identified and personal and familial cancer history of the proband

Family	Cancer history and age of proband at cancer	Number of breast cancers in the family	Breast cancers HR and HER2 status	Other cancers in relatives	Gene affected	Mutation
1	TN breast cancer, 35 yrs	1	- TN	1 pancreas, 1 thyroid cancer	RAD51D	NM_001142571.1:c.853G>A
2	TN breast cancer, 37 yrs	2	- TN - HR-HER-	1 ENT	RAD51D	NM_001142571.1:c.853G>A
3	TN breast cancer, 40 yrs	2	- TN - HR+HER-	1 CNS	MSH6	NM_000179.2:c.431G>T
4	TN breast cancer, 64 yrs	3	- TN - PR+ER-HER- - NA	1 ovarian cancer, 1 lung cancer, 1 stomach cancer	MRE11A	NM_005591.3:c.901C>G
5	TN breast cancer, 51 yrs; contralateral TN breast cancer, 63 yrs; endometrial 63 yrs, colon 68 yrs	3	- TN - TN - NA	1 endometrial, 5 colon, 1 ovarian cancer	MLH1 PALB2	NM_000249.3:c.199G>T NM_024675.3:c.2897T>C
6	TN breast cancer, 51 yrs thyroid cancer, 42 yrs; bladder cancer, 50 yrs	3	- TN - NA - NA	1 colon, 1 astrocytome, 1 retinal melanoma?	CHEK2	NM_001005735.1:c.1214G>A

NA: Not available; TN: Triple-negative breast cancer; HR: Hormone Receptor (ER+PR); ER: Estrogen Receptor; PR: Progesterone receptor; CNS: Central Nervous System; ENT: Ear, Nose and Throat; yrs: years.

one of these software was retained for further analysis.

Confirmation of variants

SNV (Single Nucleotide Variation) and indels detected using pyrosequencing within the coding sequence or intron/exon boundaries (-20 to +6) were further filtered, excluding variants recorded as polymorphisms. Variants predicted as deleterious *in silico* were confirmed by Sanger sequencing, using AmpliTaq DNA® Polymerase (Life Technologies) and BigDye® Terminator v3.1 (Life Technologies). The same primers and reagents were used to search for the confirmed variants in the control group and to analyse each proband's mutation in their relatives.

Loss of heterozygosity

Tumour tissue from mutation carriers was macrodissected from formalin-fixed paraffin-embedded tissue, and DNA was extracted from the collected cells using the Maxwell® 16 system (Promega) according to the manufacturer's instructions for formalin-fixed, paraffin-embedded samples. Primers were designed to produce small PCR products spanning the variants of interest. The mutation from each carrier was sequenced directly from a PCR product, generated using AmpliTaq DNA® Polymerase (Life Technologies), by dideoxy-sequencing using the BigDye® Terminator v3.1 and an ABI 3130XL sequencer (Life Technologies). The presence or absence of LOH (loss of heterozygosity) in tumours was determined by visual comparison of the relative size of the peaks, observed in the blood (all variants were heterozygous) and in the tumour traces.

Immunohistochemistry

Immunohistochemical detection of proteins coded by the mutated genes was performed on 4 μm-thick formalin-fixed, paraffin-embedded tissue sections of breast tumours found in the mutation carriers. Non-malignant breast tissue from a patient without any mutation was used as a control. Epitopes were retrieved by CC1 (Ventana, Tuscon, AZ) and the sections further incubated with the following antibodies: rabbit monoclonal anti-APC (LS-C49666), rabbit polyclonal anti-RAD51D ((Ala152), LS-C177138) and anti-PALB2 (LS-C288058), mouse monoclonal anti-MRE11A (LS-C53370) (all from LifeSpan Biosciences, Seattle, WA), mouse monoclonal MLH1 (clone M1, 790-4535) et MSH6 (clone 44, 790-4455) (Ventana). Antigen-antibody reaction was visualised by ultraView DAB or AP (Ventana). All procedures were performed in a fully automated immunohistochemical stainer (Benchmark XT, Ventana).

Results

Variant analysis

Seven heterozygous missense substitutions were observed in six patients, predicted as deleterious by bioinformatic analysis. All seven mutations were absent from the control group. These mutations were in six different genes: *CHEK2 NM_001005735.1:c.1214G>A* (p.Cys405Tyr); *MLH1 NM_000249.3:c.199G>T* (p.Gly67Trp); *MRE11A NM_005591.3:c.901C>G* (p.Leu301Val); *MSH6 NM_000179.2:c.431G>T* (p.Ser144Ile); *PALB2 NM_024675.3:c.2897T>C* (p.Ile966Thr); *RAD51DNM_001142571.1:c.853G>A* (p.Gly285Arg) (two cases). One, *MLH1 c.199G>T*, has been described as pathogenic, two (*MSH6 c.431G>T; RAD51D c.853G>A*) were known in genomic international databases but very rare in the general population (MAF<0.1%) and not previously associated with breast cancer; and the three others (*CHEK2 c.1214G>A; MRE11A c.901C>G; PALB2 c.2897T>C*) have not been described. One patient carried two variants, one in *MLH1* and another in *PALB2* (**Table 2**).

The *RAD51D* missense mutation c.853G>A (p.Gly285Arg) was found in two patients of different families. This amino-acid is located at the C-terminus of the AAA+ ATPase domain of the RAD51D protein, which contributes significantly to nucleotide binding [19].

We found one missense substitution in *MRE11A* (or *MRE11*), *c.901C>G* (p.Leu301Val). The amino-acid 301 is highly conserved and is located in the major functional domains of the protein: the DNA repair exonuclease domain, and the DNA-binding region.

We report a new *CHEK2* germline missense substitution, *c.1214G>A* (p.Cys405Tyr), affecting the Ser-Thr/Tyr-protein kinase catalytic domain. This amino-acid is highly conserved and there is a large physico-chemical gap

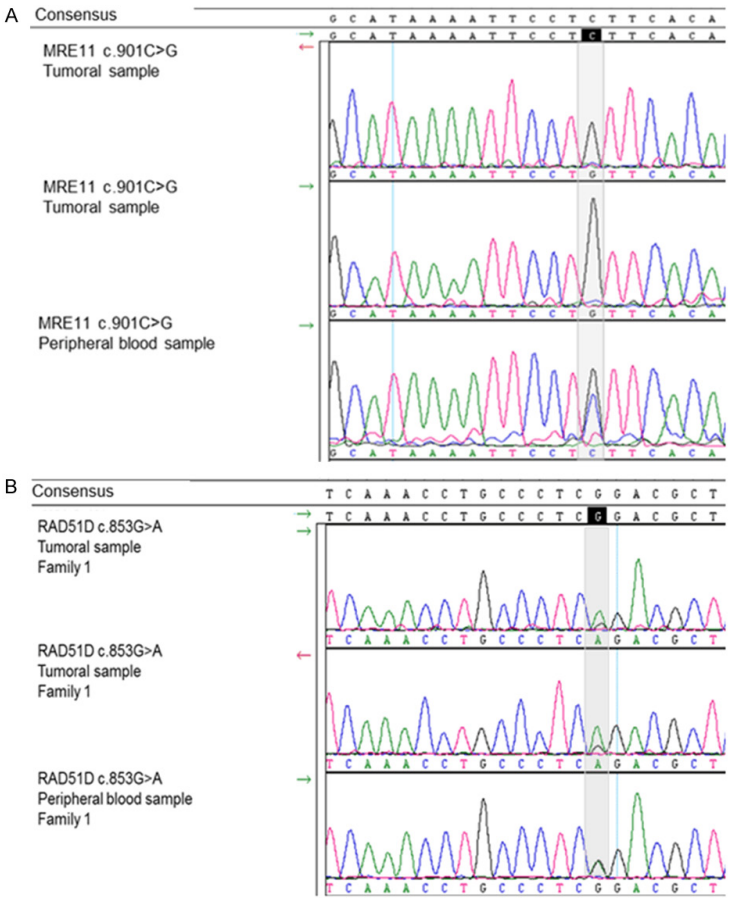

Figure 1. Sanger electropherograms representing DNA sequences of the two breast tumours with loss of the wild-type allele. A. In the peripheral blood, the patient carry the mutation MRE11 c.901C>G in the heterozygous state (last trace): 2 peaks, one blue (nulcleotide C) and one black (nulcleotide G). In the tumour (the 2 upper traces), there is no more allele with a C (blue trace) at position 901, the wild-type allele has disappeared. B. In the peripheral blood, the patient carry the mutation RAD51D c.853G>A in the heterozygous state (last trace). In the tumour, there is almost no more G at position 853. MRE11 c.901C>G and RAD51D c.853G>A loss of heterozygosity in the tumour.

between the two amino-acids. *In silico* prediction classifies this variant as disease causing. We previously observed that *in silico* predictions and *in vitro* analysis correlate well in this region of *CHEK2* [20].

PALB2 c.2897T>C missense substitutions is not localised on an identified protein activity domain, but was predicted as deleterious *in silico*.

The *MSH6 c.431G>T* substitution affects PWWP and DNA mismatch repair domains of the protein. This mutation has been already reported with an allele frequency <0.01, but its significance is unknown.

The *MLH1 c.199G>T* mutation is known to be pathogenic.

No splicing variants, truncating variants or in/dels after correction with the HECTOR software [16] were observed in the 36 genes panel.

Analysis of mutations in relatives

Following mutations in relatives of cases was possible for three of the six families. We only found a mutation in an unaffected first-degree relative of the *RAD51D c.853G>A* carrier.

A similar pattern of incomplete segregation in affected relatives has been observed for susceptibility alleles that confer modest increased risk, and reported for variants in *CHEK2, ATM, BRIP1* and *PALB2* [21-24].

Loss of heterozygosity analysis

Two of six mutations showed partial or complete loss of the wild-type allele in the tumour. Tumour material was not sufficient to perform this analysis for one of the two *RAD51D c.853G>A* carrier. Tumour DNA of the individual with the *MRE11A c.901C>G* germline mutation showed complete loss of the wild-type allele (**Figure 1A**). This complete loss of heterozygosity in the tumour suggests that this variant could be a very early event in the oncogenesis of this patient's tumour. The carrier of the *RAD51D c.853G>A* variant that could be tested showed partial loss of the wild type allele in her tumour (**Figure 1B**).

These loss of heterozygosity support that *MRE11A c.901C>G* and *RAD51D c.853G>A* are involved in tumour development according to the two-hits model of tumour suppressor genes.

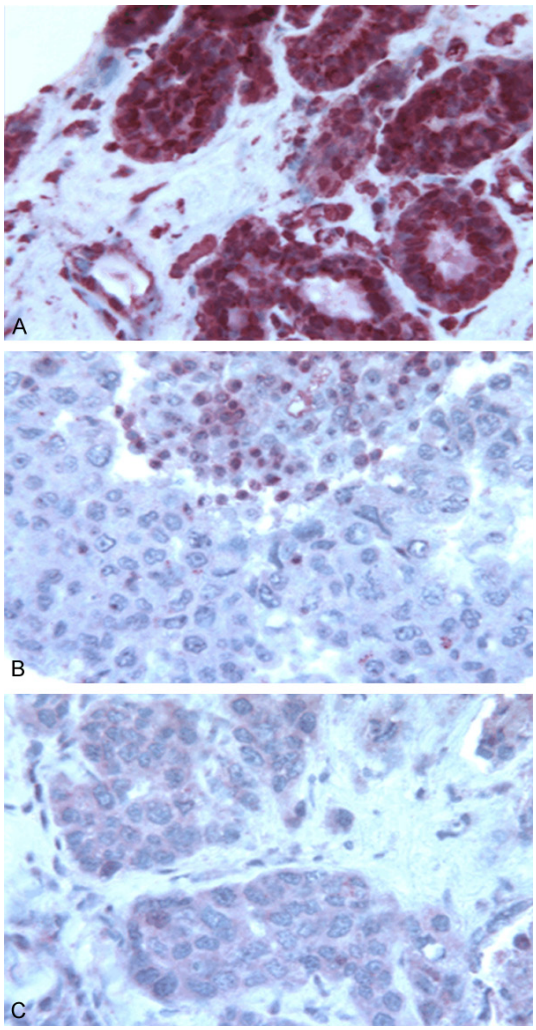

Figure 2. Immunohistochemical detection of the RAD51D protein. Formalin-fixed, paraffin-embedded tissue sections of breast tumours of the two *RAD51D* mutation carriers. A. The RAD51D protein is present as in control normal breast cells (red color in cytoplasm and nuclei). B and C. The RAD51D protein is absent in tumour cells of both mutated probands. Immunohistochemical detection of the RAD51D protein in non-malignant control breast and in tumoural tissue of mutation carriers.

Immunochemistry analysis

RAD51D protein was absent in tumour cells of both mutated probands while it was present in tumour-infiltrating lymphocytes as well as in control normal breast cells (**Figure 2**). No expression of MLH1 nor PALB2 was observed in the tumour cells nuclei of mutated cases, as compared to the control breast tissue (Figure S1). MSH6 and MRE11A staining was identical in mutated tumour and control tissues. Expression of CHEK2 could not be tested due to the small quantity of tumour tissue available.

Discussion

Germline mutations in specific genes could be responsible for triple negative breast cancer predisposition. We found seven missense mutations in 50 patients, within six different genes: *RAD51D* (two cases), *MRE11A, CHEK2, MLH1, PALB2, MSH6*. There is no previous study on familial non-BRCA1/2 triple-negative breast cancer. Literature concerns either non-familial triple-negative breast cancer [8] or familial breast cancer in general [25].

Because of a stringent selection on two criteria (familial history plus triple-negative subtype of breast cancer), our population is of limited size. However, the strength of the study has been reinforced by the homogeneity of the population.

The high number of mutations found in our work can also be explained by the choice we made to study a highly selected population as part of an initial population of familial breast cancers of 3000+ patients. Indeed, if a gene is frequently implicated in one subtype of breast cancer, and never in other subtypes, analysing the population of breast cancer in general, with all subtypes, will lead to conclude that mutations in this gene are rare or absent; however this gene could be an important predisposition factor for the small and specific subgroup of tumours where it is more frequent.

Mutations in homologous recombination DNA repair genes

We show evidence for an implication in TNBC predisposition of three genes coding important proteins of the homologous recombination (HR) DNA repair pathway, *RAD51D, MRE11* and *PALB2*.

RAD51D c.853G>A mutation is located on a key activity domain of the protein. We observed loss of heterozygosity in the tumour of one of the two carriers (the other was unavailable). Consistent with this, the RAD51D protein was absent from tumour cells of both mutated probands. *RAD51D c.853G>A* mutation was found in two individuals, and both presented a similar

phenotype, with triple-negative breast cancer at a very young age, 35 and 37 years, and with few cancers in the family (breast, thyroid and pancreatic cancers) and no ovarian cancer in the family (see Figures Family 1 and 2).

In mammalian cells, the *RAD51* paralogs are involved in homologous recombination and in maintaining genomic integrity by telomere protection [26, 27]. Mutations in *RAD51D* were mainly associated with increased risk of hereditary ovarian cancer and, although they have been observed in breast and ovarian cancer families, the association with breast cancer alone remains unclear [28-33]. A large scale study focused on triple negative breast cancer suggested that mutations in *RAD51D* could confer a risk of triple negative and basal subtypes of breast cancer specifically [8]. Finally, a case report described a 36-year old woman with a high-grade, triple negative invasive ductal carcinoma who carried a pathogenic mutation in *RAD51D* (c.556C>T; p.Arg186*) detected by 18-gene panel testing [34]. Her (non-affected) mother and her maternal aunt (medullary breast cancer at 54 years) harbored the same mutation. The phenotype of this patient was very similar to that of the two carriers of the *RAD51D* mutation from our study.

Like what we showed for the *RAD51D* mutation, the implication of *MRE11 c.901C>G* mutation in TNBC predisposition is supported by complete loss of the wild-type allele in the tumour. Immunohistochemistry revealed normal expression of MRE11A in the proband's tumour tissue. However, the epitope recognized by the antibody does not cover the mutation site, so mutated protein cannot be discriminated from wild type MRE11A. Because the mutation is in a major functional domain, the mutated protein is most likely non-functional, although ex pressed in normal quantity. Only functional studies could confirm this hypothesis. In our study, the individual carrying the *MRE11A c.901C>G* mutation had bilateral triple negative breast cancer. Her sister had ovarian cancer, her brother thyroid cancer, one nephew lung cancer and two nieces breast cancer (see Figure Family 4).

MRE11A forms a protein complex with RAD50 and NBN. This complex plays key roles in DNA double-strand break (DSB) repair, meiotic recombination, and cell cycle checkpoints, and MRE11A is also involved in maintenance of telomeres [35-37]. Mutations in *MRE11A* have been linked to Ataxia-Telangectasia-Like Disorder (ATLD) [MIM 604391]. One of the first reports of a suspected association of malignancy and mutations in *MRE11A* was about two brothers who died of pulmonary adenocarcinoma before the age of 20 [38]. One *MRE11A* mutation was also found in one case of ovarian cancer among 151 families with ovarian and breast cancer [39]. In breast cancer, evaluation of protein-truncating variants and rare missense substitutions falling in the key functional domains of the MRN complex proteins suggested a two- to three-fold increased risk of breast cancer [40]. However there is no study on *MRE11* and breast cancer of triple-negative type, but some element suggest that it could be linked. Aberrantly reduced expression of MRE11A was reported in 7% of 1000 breast tumours [41]. Moreover, among breast tumours with aberrant expression of MRE11A, 30% were triple negative. Two heterozygous mutations in *MRE11A* were found in eight patients with tumours with no or reduced expression of the three MRN complex proteins [41]. Experimentally, *MRE11A*-mutated mice exhibit extensive oncogene-induced mammary hyperplasia with frequent progression to invasive breast cancer. In addition, these mammary tumours showed largely absent ER staining and the presence of the basal-like breast cancer markers EGFR and CK5/6 in all tumours. They also exhibited hallmarks of aggressive disease, including high histopathological grade, elevated chromosomal instability and frequent development of lung metastases [42].

Finally, we found a mutation in PALB2, c.2897T>C (Ile966Thr), which is an unreported missense mutation predicted to be deleterious *in silico*. The individual carrying this mutation also harbour a *MLH1* pathogenic mutation, and presented with a TNBC at 51 years, a contralateral TNBC and endometrial cancer at 63 years, and colon cancer at 68 years. The MLH1 mutation was present in the familial branch presenting several Lynch-syndrome cancers (5 colon cancers, 1 ovarian cancer and 1 endometrial cancer) but not the PALB2 mutation (see Figure Family 5). The proband's multiple tumours may be caused by her double-heterozygote status. This hypothesis is reinforced by the absence of both PALB2 and MLH1 expression in the breast tumour. No LOH for PALB2 was found, but LOH in PALB2 breast tumours does not seem to be

a common feature of PALB2 tumour genesis [43]. Partner and localizer of BRCA2 (PALB2) is a BRCA2-interacting protein that is crucial for key BRCA2 genome caretaker functions. Monoallelic PALB2 mutations are associated with increased risk of breast and pancreatic cancer and recent studies have shown that PALB2 also interacts with BRCA1 [44, 45]. Studies performed in European populations suggest overrepresentation of triple negative (basal-like) tumours in PALB2-related breast cancers [43, 46, 47]. Heikkinen et al. [47] reported that 54.5% of breast tumours of PALB2 c.1592delT mutation carriers presented the triple negative phenotype versus 12.2% of other familial or 9.4% of sporadic breast cancer patients. Prevalence of PALB2 germline mutations in individuals with TNBC was around 1%, similar to that in familial non-BRCA1/2 breast cancer cohorts [7]. In contrast, studies in Australasian breast cancer families did not find any association between PALB2 mutations and hormone-receptor negative breast tumours [48, 49].

These results together suggest *RAD51D* mutation may predispose specifically to triple negative breast cancer. Taking into consideration a specific subtype of tumour could have revealed a susceptibility allele specific to TNBC, that need to be further explored.

There is growing evidence that *MRE11A* is a breast cancer susceptibility gene, and some data suggest that it could be particularly associated to triple negative/basal-like breast cancers.

Consistent with the fact that PALB2 is a major partner of BRCA1, and like literature begin to suggest it [43, 46, 47], our results support an implication of this gene in TNBC predisposition.

There is evidence that a substantial fraction of TNBC has HR DNA repair pathway defects [50, 51]. TNBC are characterized by extensive genomic instability [52], and deficiency in HR DNA repair is a source of genomic instability. *BRCA1*-mutated breast tumours and basal-like breast cancers show the same profile of aberrant aCGH profiles [51]. As they are HR deficient, BRCA1-mutated breast tumours can be targeted with DNA-damaging agents or PARP inhibitors. The heterogeneity of triple negative breast tumours may explain why PARP inhibitors show weak efficiency in some clinical assays[53]. Therefore treatments that seem efficient in *BRCA1*-mutated tumours should be tested in triple negative/basal-like breast tumours with germline mutation in the HR pathway (like *RAD51D* or *MRE11* genes).

Mutations in other genes

We found three mutations in three other genes, one in *CHEK2* and two in genes of the MMR (mismatch repair) group, *MSH6* and *MLH1*.

The new germline *CHEK2* missense substitution, c.1214G>A (Cys405Tyr) was not associated with LOH in the tumour, but it has been reported that tumour-specific loss of the wild-type allele is not characteristic for *CHEK2*-associated breast cancer [54]. CHEK2 functions as a homodimer: one deleteriou s allele is sufficient to produce a majority of non-functional complexes. The individual carrying this mutation presented with a multi-site personal history of cancer, with thyroid cancer at the age of 42, bladder cancer at 50, and triple negative breast cancer at 51. Her family history included another triple negative breast cancer affecting her mother at 56, a possible retinal melanoma in her maternal uncle, whose son presented with a colon cancer at 59, and another maternal cousin with an astrocytoma at 48 (see Figure Family 6). Checkpoint kinase 2 (*CHEK2*) is an important signal transducer of cellular responses to DNA damage and that acts as a tumour suppressor gene. Studies have provided evidence that *CHEK2*-truncating and/or missense mutations are associated with increased risk of breast, prostate, thyroid, colon and kidney cancers [55, 56].

This *CHEK2* mutation, *c.1214G>A,* could predispose to breast cancer, and, consistent with literature, could also be associated with a multi-site cancer predisposition. However the association of *CHEK2* mutations with triple-negative breast cancer have never been reported.

We observed a missense substitution in *MSH6*, predicted to be deleterious *in silico*, but the literature is controversial. In our study there is no strong evidence for a causal role in the breast cancer presented by the carrier: the tumour displayed no LOH and expressed MSH6 at a nor-

mal level. Although the family of the carrier is heavily affected with cancer, none belonging to the Lynch spectrum was reported. The maternal branch showed breast cancer and central nervous system (CNS) cancer (see Figure Family 3).

Several studies suggest an aside role of *MSH6* among MMR genes. Mutations of *MSH6* have been reported to be the most associated with extra-colonic cancers [57, 58].

The *MLH1 c.199G>T* was found in the individual also carrying the *PALB2* c.2897T>C mutation. This MLH1 mutation is known as pathogenic, causing Lynch Syndrome, and cancers of this mutation carrier and her family correspond to Lynch Syndrome cancer spectrum. However, TNBC could be associated with Lynch Syndrome. Immunohistochemistry of four TNBC (4/226, 1.8%) showed loss of MMR proteins (3 lost MLH1 and PMS2, and 1 lost MSH2 and MSH6); whereas none of the 90 non-triple-negative carcinomas showed the same loss [59]. Moreover, triple-negative tumours and MMR deficient colon tumours share a high genomic instability that other breast cancer subtypes don't show.

Whether breast cancer is part of Lynch syndrome tumour spectrum is controversial. This individual breast cancer could be unrelated with his MLH1 mutation, or could suggest to further explore the implication of TNBC, and not breast cancer in general, in the Lynch Syndrome cancer spectrum.

Conclusion

Familial non-BRCA1/2 TNBC seems to be associated with rare mutations in a diversity of genes, in contrast to the major-gene model typified by genes like *BRCA1* and *BRCA2*.

We report here that mutations in *RAD51D, MRE11* and *PALB2* confer a risk of triple-negative breast cancer, which could be much higher in this subtype.

This observation goes hand in hand with new approaches in breast cancer predisposition screening, especially with the recent possibilities of large-scale gene screening with high-throughput sequencing.

Identification of genes involved in triple-negative breast cancer genesis could also improve care and treatment of affected families. An approach based on genomic abnormalities starts to emerge as a substitute to the current organ-based strategy.

Looking for these mutations could be of great interest to guide therapeutic choice, and the fraction of breast cancer that should benefit from specific treatments, like PARP-inhibitor, would be larger, better identified and targeted.

Our findings have to be confirmed worldwide on larger populations of triple negative familial non-BRCA1/BRCA2 breast cancer, and it would probably be of interest to add basal markers to forthcoming studies.

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Disclosure of conflict of interest

None.

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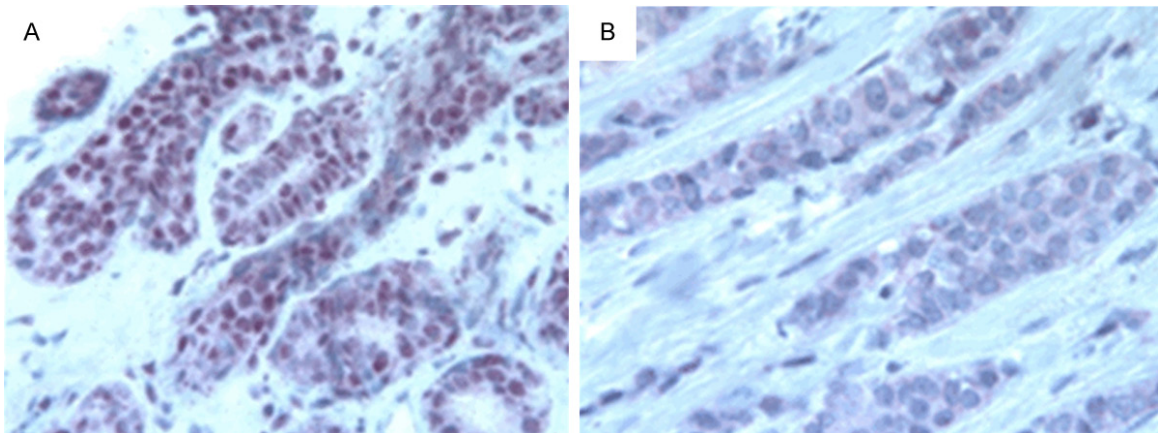
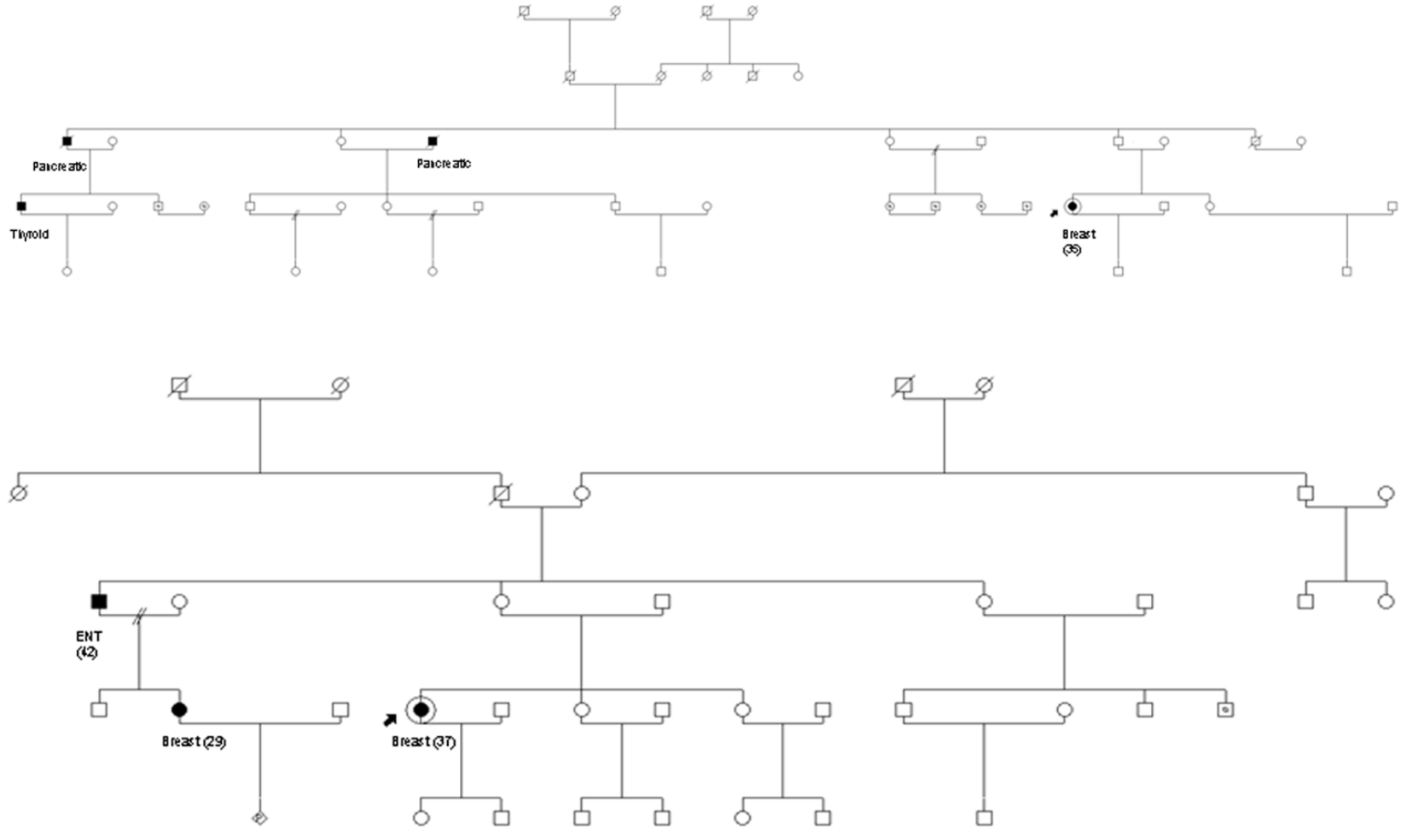


Figure S1. Immunohistochemical detection of PALB2 protein on formalin-fixed, paraffin-embedded tissue sections of non-malignant control breast and of tumoural tissue of mutation carriers. A. The PALB2 protein is present in non-malignant breast cells of an individual without *PALB2* mutation (red color). B. Absence of the PALB2 protein in tumour cells of the *PALB2* mutation carrier.


Pancreatic
Pancreatic
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Breast
(35)
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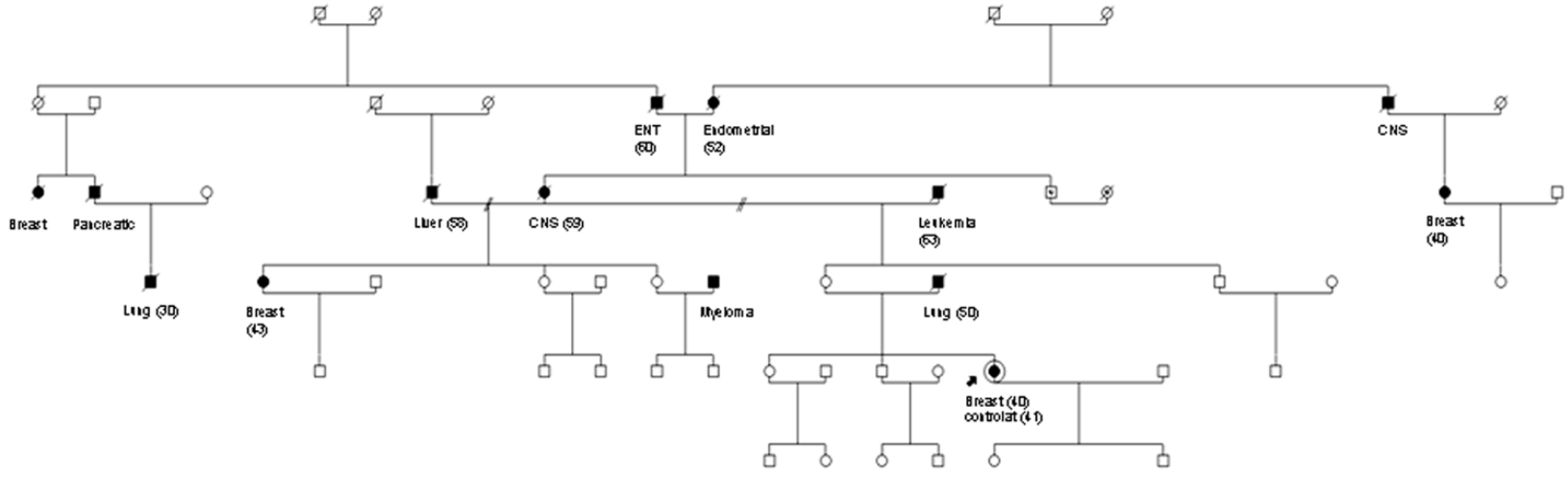
Breast (37)


ENT
(60)
Endometrial
(62)
CNS
Breast
Pancreatic
Liver (58)
CNS (59)
Leukemia
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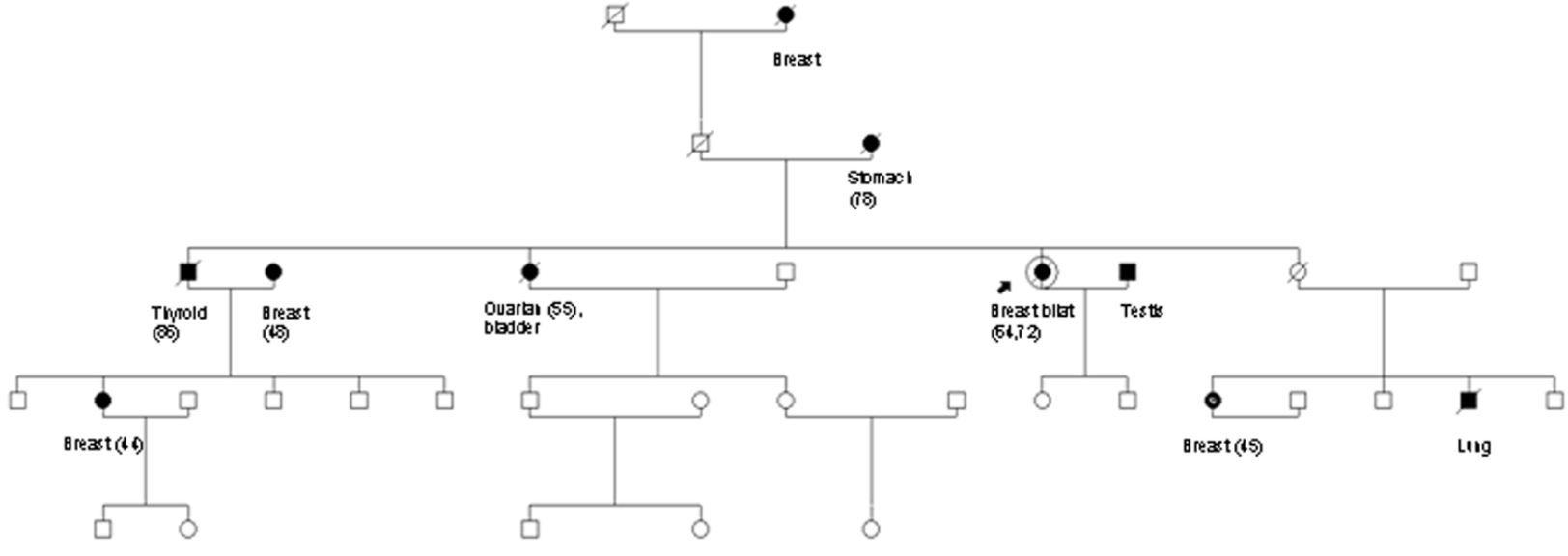
(40)
Lung (30)
Breast
(43)
Myeloma
Lung (50)
Breast (40)
controlat (41)


Breast
Stomach
(78)
Thyroid
(36)
Breast
(48)
Ovarian (55),
bladder
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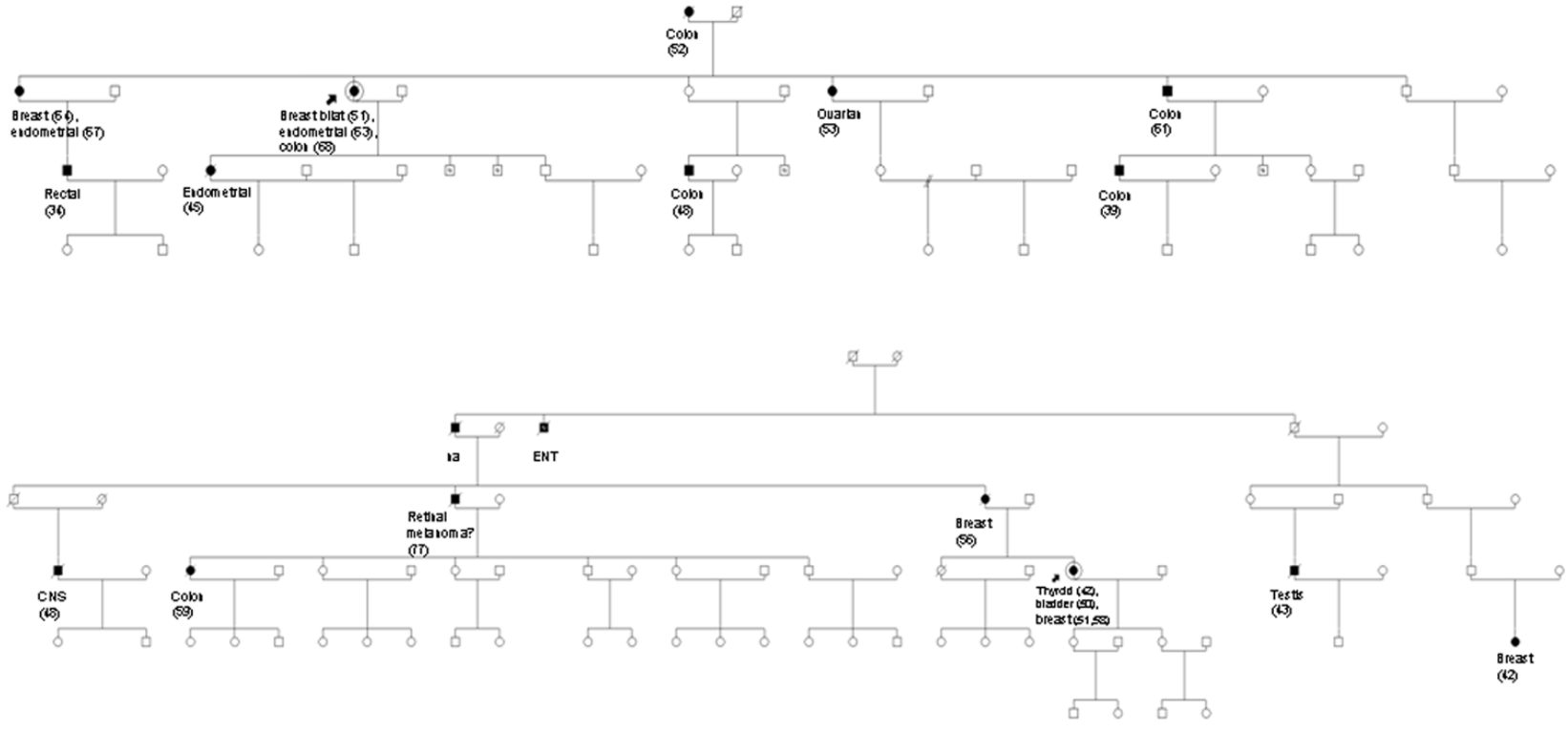
(54,72)
Testis
Breast (44)
Breast (45)
Lung



Figures Family 1-6. Genealogical trees of the seven mutated individuals. The probands are circled and indicated by an arrow. Females are represented by circles and males by squares. When they are filled with black, the individual had a cancer. Cancer types are specified below, with the age at diagnostic of cancer in brackets, when the information was available. Crossed shapes represent dead individuals. na: not available.