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Evaluation of the *BRCA1* interacting genes *RAP80* and *CCDC98* in familial breast cancer susceptibility

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Abstract *RAP80* and *CCDC98* have arisen as new candidate breast cancer susceptibility genes, since they encode for two very recently identified *BRCA1* interacting proteins. In this study we have performed the first mutational analysis of both genes in 168 multiple-case breast/ovarian cancer families, negative for mutations in *BRCA1* or *BRCA2*. We have not found truncating mutations in any of the genes and only two missense variants, p.Tyr564His in *RAP80*, and p.Met299Ile in *CCDC98* were found that could be suspected to have a pathogenic effect, although further analyses suggested that they were probably non deleterious. Our analysis suggests that *RAP80* and *CCDC98* do not play an important role as high penetrance breast cancer susceptibility genes.

Keywords Hereditary breast cancer · *RAP80* · *CCDC98* · *BRCA1*

Introduction

Only around 25% of the inherited susceptibility to breast cancer is explained by germline mutations in the known

high penetrance susceptibility genes *BRCA1* and *BRCA2* [1–3]. Mutations in genes such as *TP53* and *PTEN* have been linked with high risk for breast cancer within specific cancer syndromes [4, 5] and germline variants in other genes such as *CHEK2* [6] and *ATM* [7] and more recently the Fanconi Anemia genes *BRIP1/FANCF* and *PALB2/FANCF* [8, 9] have been found to confer modest risk to breast cancer. However alterations in these additional genes account for less than 5% of the hereditary cases and are sometimes restricted to specific populations. Despite most of the familial aggregation remains unexplained, it is worth to note that all the genes linked so far with breast cancer predisposition are functionally related and converge in a common pathway involved in the preservation of genomic integrity [10]. Therefore, analysis of other members of this pathway seems a good rationale to identify new susceptibility genes. Particularly good candidates are those known to have a direct interaction with the *BRCA* genes, as it has been the case of the last two susceptibility genes identified *BRIP1/FANCF* and *PALB2/FANCF* that interact with *BRCA1* and *BRCA2* respectively.

The *RAP80* (also known as *UIMC* Ubiquitin-Interacting Motif-Containing) and *CCDC98* (or *Abraxas*) genes encode for proteins that have been very recently identified as *BRCA1*-interactors and seem to be necessary for its role in DNA damage response [11–15]. Upon DNA damage, *BRCA1* undergoes hyperphosphorylation by the sensors *ATM*, *ATR* and *Chk2* [16–19]. *BRCA1* forms nuclear foci that co-localise with *H2AX*, a marker of DNA damage sites, and *RAD51*, implicating *BRCA1* in homologous recombination (HR) during DNA double strand break repair and meiosis [20, 21]. *RAP80* and *CCDC98* act upstream of *BRCA1*, being crucial for the formation of *BRCA1* foci in response to ionizing radiation [11–15, 22]. A model has been proposed in which *RAP80* binds to the N

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terminus of *CCDC98*, forming a stable complex that localises to damage sites depending on the UIM domain (Ubiquitin Interacting Motif) of *RAP80*; *BRCA1* is then recruited by *CCDC98* through the interaction of its C-terminal phosphorylation motif with the *BRCA1* BRCT domain [14]. Abolishing the expression of each of these three genes with short interfering RNAs (siRNAs), have demonstrated that *RAP80* is necessary for the formation of both *CCDC98* and *BRCA1* foci, while *CCDC98* is required for formation of only *BRCA1* foci [14].

Given that these two proteins are required for the correct function of *BRCA1*, the absence of any of them could lead to a similar phenotype as the caused by the inactivation of *BRCA1* itself and places *RAP80* and *CCDC98* as excellent candidate familial breast cancer susceptibility genes. In order to investigate their role in breast cancer, in the present study we have performed for the first time a molecular analysis of the *RAP80* and *CCDC98* genes in a series of 168 non-*BRCA1/2* breast cancer families.

Material and methods

Non-*BRCA1/2* families

Index cases from 168 breast cancer high-risk families recruited through the CNIO were included in the study, most of them previously reported [1, 2]. Briefly, families contained at least three females affected with breast cancer or at least two first-degree females affected with breast cancer (at least one of them diagnosed before 50) or at least one case of female breast cancer and at least one case of either ovarian, female bilateral breast or male breast cancer. DNA extracted from peripheral blood of the 168 cases, had been fully screened for mutations in *BRCA1* and *BRCA2* by a combination of DHPLC and direct sequencing and found to be negative.

Controls

DNA samples from 855 women aged between 25 and 65 years and without personal or familial antecedents of any cancer were analyzed to establish the frequency in the general population of some of the variants found in *RAP80* and *CCDC98* genes.

Molecular analysis of *RAP80* and *CCDC98* genes

DNA from the 168 index cases was subjected to Whole Genome Amplification by using the GenomiPhiV2 Amplification Kit (GE Healthcare UK Limited, Buckinghamshire,

UK) according to manufacturer's instructions. Primers were designed to amplify the whole coding sequence, exon-intron boundaries and part of the 5' and 3' untranslated regions (UTR) of *RAP80* and *CCDC98*, using the program Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). 29 primer pairs were used to cover the 14 coding exons of *RAP80* (Gen-bank NM_016290) and 9 coding exons of *CCDC98* (Gen-bank NM_139076) (Supplementary Table 1). PCR-products were analysed by DHPLC on the WAVE HT system (Transgenomic, Omaha, NE) using an acetonitrile gradient and scrutinised for aberrant profiles with the NavigatorTM software (Transgenomic, Omaha, NE). Samples representative of each DHPLC pattern were bidirectionally sequenced using the Big Dye Terminator Cycle sequencing kit and a 3730 automated sequencer (ABI Perkin Elmer). All non-described variants were confirmed by sequencing a fresh aliquot of the non-WGA stock DNA. Those missense variants found in either gene that were not reported in the <http://www.ncbi.nlm.nih.gov/SNP/>, were analysed by DHPLC in the 855 control samples to investigate whether they were common polymorphisms and establish their frequency in our population.

Immunohistochemistry for DNA repair proteins detection.

Five millilitres of peripheral blood was collected from three females (two carriers and one non-carrier) of the family harbouring the variant p.Met299Ile in *CCDC98*. Lymphocytes were extracted using Histopaque 1077 (Sigma Aldrich). After centrifugation at 1,500 rpm for 30 min, lymphocytes were collected and grown for 24–48 h in RPMI 1640 medium and stimulated with 5 µg/ml phytohaemagglutinin PHA (Invitrogen). Cells were then non-irradiated or irradiated with 10 Gy incubated for 2 h after irradiation and spun down onto microscope slides. Cells were fixed with 2% paraformaldehyde in PBS 0.5% Triton X-100 for 15 min. To visualize the DNA-damage induced focus formation, staining was done incubating 10 min with primary antibodies anti phospho-H2AX (Upstate, clone JBW301) and *CCDC98* (kindly provided by Dr J Chen). After incubation with the corresponding secondary antibody, lymphocyte nucleuses were stained with 0.1 µg/ml DAPI (Invitrogen) for 2 min at room temperature.

Results

Screening of the *RAP80* gene

The whole coding sequence and exon-intron boundaries of the *RAP80* gene were analysed in the index cases of the 168

Table 1 Genetic variants identified in the *RAP80* gene

Nucleotide change ^a	Protein change	rs number ^b	Frequency of heterocigotes ^c	Pathogenicity ^d
5' UTR 160A > G	–	–	0.02	No
c.43 C > T	p.Trp15Arg	rs13167812	0.01	No
c.228T > C	p.Ile76Ile	rs1700490	0.472	No
c.1677–39C > T	–	–	–	No
c.1344A > C	p.Thr448Thr	rs365132	0.463	No
c.1686G > A	p. Lys562Lys	–	–	No
c.1690T > C	p. Tyr564His	–	<0.001	Unknown
c.1849–17A > G	–	rs 2292255	0.442	No
c.1913–86A > G	–	–	0.05	No
c.1913–78A > G	–	rs2292256	0.442	No

^a Mutation nomenclature listed uses GenBank reference sequences NM_016290.3 and NP_057374 .3 with numbering starting at the A of the first ATG, following the journal guidelines (www.hgvs.org/mutnomen) except for the variants located in the UTR for which the numbering starts at the first nucleotide of the mRNA sequence

^b The rs number is indicated for those SNPs previously reported in the NCBI database <http://www.ncbi.nlm.nih.gov/SNP/>. These variants were considered as common polymorphisms or at least not pathogenic variants

^c Frequency based on the number of heterocigotes found within the 168 cases analysed. Frequencies in bold are those established by analyzing the corresponding variant in the 855 control individuals

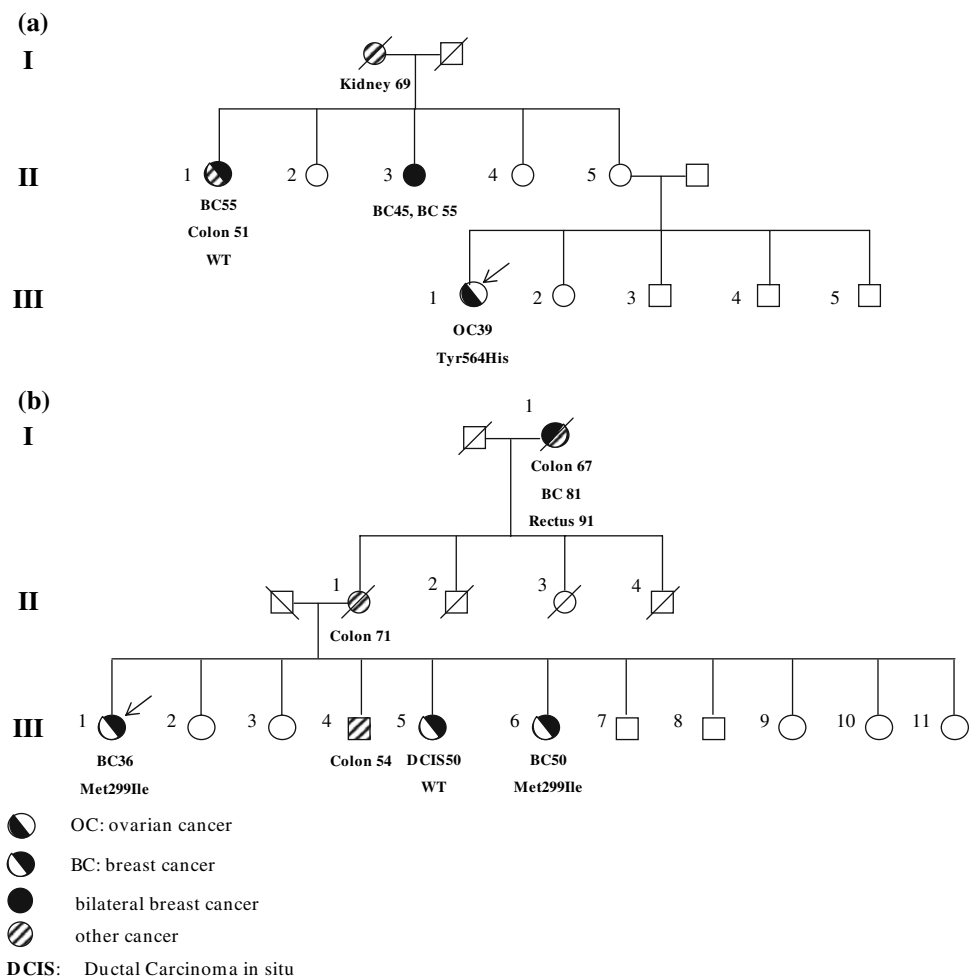
^d All the variants already reported in the NCBI database were considered as not pathogenic, as well as those located in introns and not affecting the consensus acceptor or donor splice sites or those that resulted in a synonymous change

non-BRCA1/2 families. During the screening we identified 10 different variants (Table 1), half of which had been already described in the NCBI SNP database (<http://www.ncbi.nlm.nih.gov/SNP/>) and were considered as common polymorphisms. We found five not previously described variants, four of which, 5' UTR 160A > G, c.1677–39C > T, c.1913–86A > G and p. Lys562Lys were considered as non-pathogenic, as the first one was located in the 5' UTR, two were located in introns not affecting to the consensus acceptor splice site and the last one in exon 12 represented a synonymous change. The fifth not previously described variant, p.Tyr564His located in exon 12, was the only one that could be suspected to have a pathogenic effect, given that represented a non-synonymous change, located in the second zinc finger-like motif of the protein, in a position that is highly conserved across different species (<http://www.ncbi.nlm.nih.gov/blast/>). To further investigate its potential role in breast cancer susceptibility, we estimated the frequency of Tyr564His in the general Spanish population, performing a screening by DHPLC in 855 controls. None of the control samples harboured the variant, confirming that it was not a common polymorphism. The variant was found in a woman affected with ovarian cancer at 39 years of age (Fig 1a, III-1), who had one maternal aunt affected with colon and breast cancer at the ages of 51 and 55 respectively and another maternal aunt diagnosed with bilateral breast cancer at the ages of 45 and 55 (Fig. 1a). We could analyse the DNA sample from the woman affected with colon and breast cancer (Fig 1a, II-1) but she did not carry the Tyr564His variant.

Screening of the *CCDC98* gene

As for *RAP80*, the whole coding sequence and exon-intron boundaries of the *CCDC98* gene were analysed in the index cases of the 168 non-BRCA1/2 families. During the screening we identified 6 different variants (Table 2), those described in the NCBI SNP database (<http://www.ncbi.nlm.nih.gov/SNP/>) being considered as polymorphisms. We found four not previously described variants, three of which, p.Thr141Ile, c.542–30G > A and 3' UTR 1696T > C were considered as non pathogenic, the first one because despite representing a non-synonymous change was found with a frequency of heterocigotes of 0.02 in a control sample of 855 individuals and the other two because they were located in intron 5 (not affecting to the consensus acceptor splice site) and 3' UTR respectively. The forth not previously described variant, p.Met299Ile located in exon 9, was the only one that could be suspected to have a pathogenic effect, given that represented a non-synonymous change and was found only in one of the 168 index cases and in none of the 855 controls. The affected amino-acid was not within the N-terminal or C-terminal regions known to be necessary for the interaction of *CCDC98* with *RAP80* and *BRCA1* respectively, however the position is highly conserved across different species (<http://www.ncbi.nlm.nih.gov/blast/>). The variant was found in a woman affected with invasive breast cancer at 36 years of age (Fig 1b, III-1), who had two sisters affected with ductal carcinoma in situ (DCIS) and invasive breast cancer respectively both at the age of 50 (Fig. 1b, III-5 and

Fig. 1 (a) Summarized family history of the index case (indicated by an arrow) harbouring the Tyr564His variant in *RAP80*. Coloured-in circles represent individuals with breast or ovarian cancer, individuals with other cancers are indicated by circles with diagonal lines and type of cancer indicated. The age at diagnosis is indicated under each individual. (b) Family harbouring the variant Met299Ile in *CCDC98*



III-6). Blood samples from the two affected sisters could be obtained and their DNA analysed for the presence of the Met299Ile alteration, finding that only the one affected with invasive breast cancer carried the variant while the one affected with DCIS did not (Fig 1b, III-5 and III-6). In this case, given the availability of material from the three affected sisters, immunohistochemistry experiments were performed in order to investigate if there were any difference regarding the presence of CCDC98 in the DNA-damage induced focus upon DNA damage between the women carrying the variant and the one that did not (see Material and Methods). CCDC98 localized normally to nuclear foci lymphocytes in the three patients analyzed.

Discussion

Genes encoding for proteins that interact directly with BRCA1 and/or BRCA2 or participate in the DNA damage response are good candidates to represent additional breast cancer susceptibility genes. Several of these genes have already been investigated and a few of them such as

CHEK2, *ATM*, *FANCI/BRIP1* and *FANCN/PALB2* (the last two directly interacting with *BRCA1* and *BRCA2* respectively) have been found to confer modest risk to the disease [6–9]. The Ubiquitin-Interacting Motif-Containing Protein RAP80 and the coiled-coil domain-containing protein CCDC98 are two very recently identified BRCA1 associated proteins and seem to be essential for the formation of BRCA1 foci upon DNA damage [11–15]. The fact that these two genes act upstream *BRCA1* and are necessary for its recruitment to DNA damage sites [15], make them specially good candidates as breast cancer susceptibility genes, given that the absence of any of them could lead to a phenotype similar to that caused by the absence of *BRCA1*.

In the present study we have investigated for the first time, the role of *RAP80* and *CCDC98* genes in breast cancer susceptibility by analysing their entire coding sequence in a series of 168 non-*BRCA1/2* breast/ovarian cancer families. In total we have identified 10 and 6 different variants in *RAP80* and *CCDC98* respectively, none of them giving rise to stop codons. Around half of the variants (5 in *RAP80* and 2 in *CCDC98*) had been already reported in the NCBI SNP database (<http://www.ncbi.nlm.nih.gov/SNP/>) and were

Table 2 Genetic variants identified in the *CCDC98* gene

Nucleotide change ^a	Protein change	rs number ^b	Frequency of heterocigotes ^c	Pathogenicity ^d
c.422C > T	p.Thr141Ile	–	0.02	No
c.542–30G > A	–	–	0.01	No
c.897G > A	p.Met299Ile	–	<0.001	Unknown
c.1042G > A	p.Ala348Thr	rs12642536	0.431	No
c.1117G > A	p.Asp373Asn	rs13125836	0.115	No
3' UTR 1696T > C	–	–	0.01	No

^a Mutation nomenclature listed uses GenBank reference sequences NM_139076 and NP_620775 with numbering starting at the A of the first ATG, following the journal guidelines (www.hgvs.org/mutnomen) except for the variants located in the UTR for which the numbering starts at the first nucleotide of the mRNA sequence

^b The rs number is indicated for those SNPs previously reported in the NCBI database <http://www.ncbi.nlm.nih.gov/SNP/>. These variants were considered as common polymorphisms or at least not pathogenic variants

^c Frequency based on the number of heterocigotes found within the 168 cases analysed. Frequencies in bold are those established by analyzing the corresponding variant in the 855 control individuals

^d All the variants already reported in the NCBI database were considered as not pathogenic, as well as those located in UTRs or introns (not affecting the consensus acceptor or donor splice sites), those that resulted in a synonymous change or those non-synonymous changes found to have a frequency of heterocigotes >1% in the control population

considered as common polymorphisms (Tables 1 and 2). From the not previously reported variants, those located in introns (not involving consensus splice sites), UTRs or representing silent changes were considered as not likely to be pathogenic (Tables 1 and 2). Three missense not previously reported alterations were found, Tyr564His in *RAP80*, and Thr141Ile and Met299Ile in *CCDC98*. From these, Thr141Ile in *CCDC98* was found with a frequency of 0.02 of heterocigotes in a control sample of 855 individuals and therefore considered as a common polymorphism.

The amino-acid changes Tyr564His in *RAP80* and Met299Ile in *CCDC98* were the only variants further investigated as putatively involved in breast cancer susceptibility, as they were found each in one of the 168 index cases and in none of the 855 controls and could potentially affect the functionality of the respective proteins. Tyr564His in *RAP80* is located outside the recently identified Abraxas-interacting domain or the UIM motifs, necessary for the localization of *RAP80-CCDC98* complex in the DNA damage induced nuclear foci [14, 23]. Met299Ile in *CCDC98* is outside the N-terminal or C-terminal regions known to be necessary for its interaction with *RAP80* and *BRCA1* respectively [12, 14, 23]; however, just by their position, we could not rule out that these changes could have any effect on the functionality of the proteins, especially taking in account that it is thought that multiple regions in both proteins are required for the interaction between *RAP80* and *CCDC98* [15]. Actually, both missense variants were located in highly conserved positions and in the case of Tyr564His in *RAP80*, the change was located in the second zinc finger-like motif of the protein, whose exact function is not known, but is a type of motif usually involved in DNA recognition and protein-protein interactions.

Analysis of the families in which the two variants were identified was more revealing, as none of them were found to show a complete segregation with the disease. In the case of Tyr564His in *RAP80*, only one of the two affected relatives of the index case, could be analyzed for the presence of the variant and was found not to carry it (Fig. 1a), which suggested that at least it could be ruled out as a high penetrance allele. For the p.Met299Ile in *CCDC98*, the analysis was more complete, given that blood samples from the two affected sisters of the index case could be obtained and their DNA analysed for the presence of the Met299Ile alteration. Only one of the sisters was found to carry the variant while the other did not (Fig. 1b). Although again the segregation was not total, from the three affected sisters (considering the index case), the only non carrier was the one affected with DCIS. In the case of *BRCA1/2* families, there is controversy about the consideration of DCIS as a criterion for *BRCA* risk assessments, given that a diagnosis of DCIS seem not be as suggestive for the presence of a underlying *BRCA* mutation than a diagnosis of IBC [24, 25]. The same could be applicable to the case of a potential new susceptibility gene, which made in our case, the segregation analysis of the variant in *CCDC98* difficult to interpret. Further investigation of the variant was performed by analysing the nuclear *CCDC98* foci formation in lymphocytes from the three affected sisters. Immunohistochemistry analysis revealed that, upon DNA damage prompted by ionizing radiation, both wild-type and Met299Ile harbouring *CCDC98* localized normally in the nuclear foci, suggesting that this variant is not affecting to the correct localization of *CCDC98* in the DNA breaks sites. Therefore segregation and immunohistochemistry analyses both suggested that the Met299Ile variant in *CCDC98* is not a high susceptibility allele either.

In summary, this first screening of the *RAP80* and *CCDC98* genes in 168 index cases from non-BRCA1/2 families, suggests that they can be ruled out at least as high penetrance breast cancer genes. However, we can not exclude the possibility that allelic variants in these two genes could be associated with a low or modest risk to the disease similar to the found for other genes [6–9].

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