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Identification of a common polymorphism in the *TopBP1* gene associated with hereditary susceptibility to breast and ovarian cancer

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ABSTRACT

Besides BRCA1 and BRCA2 other genes are also likely to be involved in hereditary predisposition to breast and/or ovarian cancer. TopBP1 (topoisomerase IIβ binding protein 1) displays sequence homology as well as functional similarities with BRCA1, and the two proteins have been suggested to function partly in the same cellular processes. TopBP1 is crucial for DNA damage and replication checkpoint controls. Based on its biological significance, we reasoned that *TopBP1* is a plausible susceptibility gene for hereditary breast and/or ovarian cancer and therefore screened affected index cases from 125 Finnish cancer families for germline changes by conformation sensitive gel electrophoresis (CSGE). Altogether 19 different sequence alterations were detected. A novel heterozygous Arg309Cys variant was observed at elevated frequency in the familial cancer cases compared to healthy controls (15.2% versus 7.0%; $P = 0.002$). Current results suggest that Arg309Cys is a commonly occurring germline alteration possibly associated with a slightly increased breast and/or ovarian cancer risk. This is the first study reporting mutation screening of the *TopBP1* gene in a familial cancer material.

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1. Introduction

Breast and ovarian cancers are among the most common malignancies of women in Western countries.[1] About 5–10% of the cases are considered familial,[2] and 40–50% of them can currently be explained by mutations in two main susceptibility genes, *BRCA1* and *BRCA2*.[3–5] Of the remaining cases no more than 5% are caused by defects in other studied genes, such as *TP53*, *PTEN*, *ATM* and *CHK2*.[6–10] Therefore, additional susceptibility genes are likely to be involved. The remaining familial aggregation of breast cancer may be explained by a polygenic model, in which a large number of susceptibility alleles together are responsible for the disease.[11,12] However, due to genetic heterogeneity, the identification of additional genes has proved difficult. As most of the known cancer susceptibility genes encode proteins involved in the monitoring of genome integrity, it is expected that potential candidate genes could also be ascribed similar functions.

TopBP1 was originally identified through its association with DNA topoisomerase IIβ, and like its homologues in yeast

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(*Schizosaccharomyces pombe* Rad4/Cut5 and *Saccharomyces cerevisiae* Dpb11 proteins) and fly (*Drosophila melanogaster* Mus101 protein) it is involved in DNA damage and replication checkpoint pathways.[13–16] TopBP1 possesses eight BRCT (BRCA1 C-terminal) domains that have been identified in a number of proteins involved in DNA repair and cell cycle regulation.[17,18] The C-terminal region of TopBP1 containing two BRCTs shows considerable similarity to the corresponding part of BRCA1.[13,14] Besides the sequence homology, TopBP1 also shares many other features with BRCA1. The expression of both proteins is highest in S-phase cells, and in response to DNA damage or replication blocks both TopBP1 and BRCA1 are phosphorylated by ATM and co-localise to sites of DNA damage at IR induced nuclear foci or arrested replication forks, respectively.[14,15,19] The localisation patterns of TopBP1 and BRCA1 have similarities also during late mitosis as well as in meiotic prophase I.[20] Furthermore, the two proteins have been shown to possess overlapping functions in G2/M checkpoint regulation and to be required for activation of CHK1, a downstream substrate of ATR, in response to DNA damage.[21] In intact cells TopBP1 associates with ubiquitin ligase hHYD, and ubiquitination leads to degradation of the protein through the proteosome pathway.[22] IR-induced phosphorylation inhibits TopBP1 ubiquitination stabilising the protein and stimulates its co-localisation with BRCA1 and several other molecules critical for DSB DNA repair, including the immediate DNA damage marker H2AX, PML, Rad50, ATM, Rad9, BLM, NBS1 and 53BP1.[15,22,23] Expression studies have shown that TopBP1 is required for normal cell survival, since down-regulation of TopBP1 results in reduced cell viability due to increased apoptosis.[15]

The biological function of TopBP1 and its close relation with BRCA1 prompted us to investigate whether germline alterations in the *TopBP1* gene are associated with an increased hereditary risk of developing breast and/or ovarian cancer. To evaluate this possibility, we screened the whole coding sequence and intron–exon boundaries of *TopBP1* in affected index cases from 125 Finnish cancer families.

2. Materials and methods

2.1. Study subjects and controls

In total, 125 breast and/or ovarian cancer families originating from Northern Finland were selected for mutation analysis of the *TopBP1* gene. Of the studied families, 92 were associated with breast, 30 with breast-ovarian and 3 with ovarian cancer. Inclusion criteria for the 75 high-risk families were three or more cases of breast and/or ovarian cancer in first- or second-degree relatives, or two cases if at least one of them showed a high-risk feature such as early disease onset ($\leqslant$35 years), bilateral breast cancer or multiple primary tumours. Most of the high-risk families displayed three or more cancer cases. The remaining 50 families with moderate disease susceptibility displayed two cases of breast and/or ovarian cancer in first- or second-degree relatives. All of the high-risk families had previously been tested for germline mutations in *BRCA1*, *BRCA2*, *CHK2* and *TP53*,[24–26] and 10 families showed alterations in *BRCA1* or *BRCA2*. All patients participating in the study signed an informed consent for obtaining pedigree data and blood specimens for genetic analyses. Testing was carried out on DNA from an index case, defined as the youngest available breast or ovarian cancer patient in the family. The population controls consisted of DNA samples from 697 anonymous cancer-free blood donors collected through the Finnish Red Cross Blood Transfusion Service. The occurrence of *TopBP1* Arg309Cys was also tested in 187 breast cancer patients without known family history of the disease. Both reference groups (not tested for *BRCA1* or *BRCA2* mutations) originated from the same geographical region as the studied cancer families. The study has been approved by the Ethical Board of the Northern Ostrobothnia Health Care District and the Finnish Ministry of Social Affairs and Health.

2.2. Mutation screening and sequence analyses

Genomic DNA was extracted from blood lymphocytes using either the standard phenol–chloroform protocol or the Puregene D-50K purification kit (Gentra, Minneapolis, MN, USA). The complete coding sequence (GenBank: Q92547 protein; NM_007027.2 mRNA; 1522 amino acids) and adjacent intronic regions of *TopBP1* were screened for germline mutations by CSGE, which is an effective and cost-efficient procedure to screen for sequence variation with high sensitivity and specificity.[27,28] All positive screening results were confirmed by re-amplification of the original genomic DNA sample and by direct sequencing using the SequiTherm EXCEL™II DNA Sequencing Kit-LC (Epicentre Technologies, Madison, WI, USA) and the Li-Cor IR^2 4200-S DNA Analysis system (Li-Cor, Lincoln, NS, USA). Amplifying primers were designed using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) utilising sequence information obtained from publicly available databases (GenBank: NT_022393). In order to detect variant homozygotes for the possible disease related Arg309Cys alteration, PCR followed by restriction fragment analysis was utilised. The identification of wild-type (C/C) and variant (T/T) homozygotes at position 1010 was based on amplification of a 383-bp DNA fragment digested with *TatI* (Fermentas, Burlington, ON, Canada). Oligonucleotide sequences and reaction conditions for PCR and restriction enzyme digestion are available upon request.

2.3. Statistical analyses

The observed differences in mutation frequencies between the hereditary or sporadic group of breast cancer patients and control samples were analysed by Pearson's Chi-Square or Fisher's exact test. All *P*-values were two-sided. Statistical analyses were carried out with SPSS version 12.0 for Windows (SPSS, Chicago, IL, USA).

2.4. Cell culture and western blot analysis

Two wild-type and four *TopBP1* Arg309Cys heterozygous lymphoblastoid cell lines (LCLs) were derived from patients diagnosed with breast cancer. Cells were cultured in RPMI 1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum, 1% L-glutamine and gentamycin (10 μg/ml) at 37 °C in a 5% CO_2 atmosphere. Cells were centrifuged,

washed twice with PBS and the pellet was suspended with 2.5 × Laemmli buffer and sonicated briefly. The protein concentration was measured using the RC DC protein assay kit (BioRad, Hercules, CA, USA). Twenty-five micrograms of protein was separated by SDS-PAGE, after which the gels were stained with Sypro Orange (BioRad, Hercules, CA, USA) to verify equal loading and electroblotted onto PVDF membranes (Millipore, Billerica, MA, USA). The rabbit polyclonal α-TopBP1.2 antibody has been described previously[14] and peroxidase-conjugated goat α-rabbit was from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Secondary antibody was used at dilutions of 1:10 000 to 1:15 000. Signals were detected by chemiluminescence, using the SuperSignal West Femto detection kit (Pierce, Rockford, IL, USA). ImageQuant 5.2 (Amersham Lifesciences, Toronto, ON, Canada) was used for quantification of densitometrically scanned western blot signals. Analyses were performed in triplicate.

3. Results

The present study of 125 Finnish breast and/or ovarian cancer families revealed, altogether, 19 germline alterations in the *TopBP1* gene (Tables 1 and 2). Ten of the observed alterations occurred in exon regions, three of which were novel while the rest have been previously reported in the single nucleotide polymorphism (SNP) database. The locations of the identified amino acid changes are shown in Fig. 1. Four novel and five previously known variants were observed in intron regions. In order to evaluate the pathogenicity of the observed variants, the frequencies of all exonic and intronic changes were compared between patients and healthy control individuals. Furthermore, all alterations were assessed for possible effects on splice site consensus sequences (http://www.fruitfly.org/seq_tools/splice.html), and coding sequence variants were tested by the ESEfinder 2.0 program (http://rulai.chsl.edu/tools/ESE/) to ascertain whether the observed variants fell within predicted exonic splicing enhancer (ESE) sequences and whether they would affect ESE functions.

Of the novel exonic alterations, two were missense (1010C > T, Arg309Cys; 3123G > A, Arg1013Gln) and one resulted in a synonymous change (4336A > G, Ser1417Ser). The incidence of Arg309Cys among the familial cancer cases was found significantly elevated when compared to healthy controls, indicating that the variant could possibly be disease-related. The nucleotide substitution leading to Arg309Cys showed no effect on binding sites of exonic splicing enhancers, but was located within a consensus splicing sequence and predicted to affect the consensus acceptor site. To further evaluate the mutational status of the Arg309Cys variant, DNA samples from altogether 697 healthy controls and 187 breast cancer cases without known family history of the disease were analysed. The observed frequencies for C/T heterozygotes were 15.2% (19/125) in the familial breast and/or ovarian cancer cases compared to 7.0% (49/697) in the controls ($P = 0.002$; odds ratio [OR] = 2.4; 95% confidence interval [CI] = 1.3–4.2). In contrast to the familial cases, the frequency for breast cancer patients without known family history of the disease (9.1%; 17/187), but was not significantly higher than in controls ($P = 0.341$; OR = 1.3; 95% CI = 0.7–2.4). The other novel missense variant Arg1013Gln was predicted to have an effect on an ESE motif, but similar frequencies both in familial cases and controls suggested that it is likely to represent a harmless polymorphism. Moreover, the novel A to G transition at position 4336 was absent from the controls, but as it resulted in a synonymous change (Ser1417Ser) and did not have any effect on predicted splicing consensus sequences or ESE motifs, it was unlikely to be a pathogenic alteration.

TopBP1 Arg309Cys was present in 19 of 125 familial index cases. To further study the role of Arg309Cys in breast cancer predisposition, available affected and unaffected family members were tested for mutation status. Additional samples were obtained from only eight of 19 families. From four families one additional sample from a breast cancer patient was available, and in all of these families the other women diagnosed with breast cancer also carried the Arg309Cys allele. In the remaining four families, both affected non-carriers and healthy carriers were observed. Due to the small number of additional DNA samples, the segregation of Arg309Cys with the disease remained unclear. However, based on available data, the segregation of Arg309Cys with breast cancer appeared to be incomplete at least in some of the families in which it was detected.

Table 1 – Sequence variation observed in the protein-encoding regions of the *TopBP1* gene

Exon	Nucleotide change	Effect on protein	Frequency[a]		P	Status
			Cases	Controls		
Ex8	1010C > T	Arg309Cys	15.2% (19/125)	7.0% (49/697)	0.002	Novel variant
Ex10	1454A > C	Lys457Gln	33.6% (42/125)	39.2% (116/296)	0.279	Known variant[c]
Ex12	1996T > C	Val637Val	20.8% (26/125)	21.1% (19/90)	0.956	Known variant[c]
Ex14	2535C > T	Ser817Leu	26.4% (33/125)	27.7% (155/559)	0.764	Known variant[c]
Ex18	3097G > A	Val1004Val[b]	3.2% (4/125)	3.5% (11/311)	1.000	Known variant[c]
Ex18	3123G > A	Arg1013Gln	12.0% (15/125)	10.9% (34/311)	0.750	Novel variant
Ex19	3210A > G	Asn1042Ser	36.0% (45/125)	38.2% (34/89)	0.742	Known variant[c]
Ex25	4163C > T	Leu1360Leu	35.2% (44/125)	34.1% (31/91)	0.863	Known variant[c]
Ex26	4336A > G	Ser1417Ser	0.8% (1/125)	– (0/362)	0.257	Novel variant
Ex27	4432A > G	Ser1449Ser	22.4% (28/125)	22.0% (20/91)	0.941	Known variant[c]

a Heterozygotes.
b Always observed together with Arg1013Gln.
c Reported in the SNP database.

Table 2 – Sequence variation observed in the intron regions of the *TopBP1* gene

Location	Nucleotide change	Frequency[a]		Status
		Cases	Controls	
IVS1 + 177	C > G	8.0% (10/125)	10.0% (3/30)	Known variant[c]
IVS2 − 48	T > A	7.2% (9/125)	8.8% (6/68)	Known variant[c]
IVS15 − 27	A > G[b]	22.4% (28/125)	23.9% (21/88)	Novel variant
IVS15 + 16	T > C	8.8% (11/125)	8.0% (7/88)	Novel variant
IVS17 − 28	A > G[b]	22.4% (28/125)	24.7% (23/93)	Novel variant
IVS22 + 27	C > G[b]	22.4% (28/125)	25.0% (23/92)	Known variant[c]
IVS22 + 44	T > C[b]	22.4% (28/125)	25.0% (23/92)	Known variant[c]
IVS23 − 18	G > A	35.2% (44/125)	35.2% (32/91)	Known variant[c]
IVS28 − 50	C > T	0.8% (1/125)	0.5% (1/182)	Novel variant

IVS, intervening sequence.
a Heterozygotes.
b Observed together.
c Reported in the SNP database.

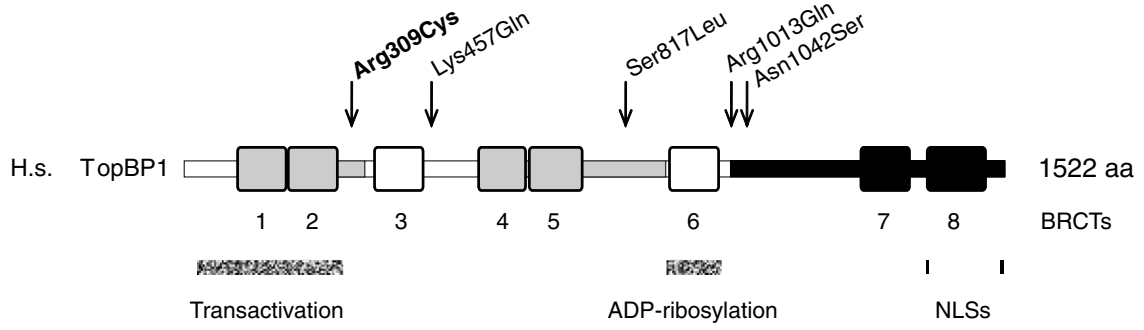

Fig. 1 – Locations of amino acid changes in the human TopBP1 protein caused by non-synonymous changes observed in the *TopBP1* gene. The changes are marked above the schematic protein structure and the possible breast cancer related Arg309Cys is indicated in bold. BRCT domains are boxed: black indicates similarity to the C-terminal region of BRCA1 and grey to the N-terminal region of fission yeast TopBP1 homolog Cut5. Additional functional domains are shown below the diagram; NLS stands for nuclear localisation signal.

None of the index cases carrying *TopBP1* Arg309Cys belonged to the 10 *BRCA1* or *BRCA2* mutation-positive families. The frequency of Arg309Cys among familial cases was also examined according to family history of breast and/or ovarian cancer and to number of cancer cases in the family. The prevalence appeared to be similar among patients having either a family history of breast or breast/ovarian cancer (18.2% [6/33] versus 14.1% [13/92]; $P = 0.578$). The distribution of carriers between high- and moderate-risk families was also equal. In order to also identify individuals homozygous for the variant allele (T/T), restriction enzyme analysis was performed in familial and control groups. In the familial material one T/T homozygous individual diagnosed with breast cancer at age 45 was observed. The only additional sample from the same family was from a paternal aunt, who had breast cancer at age 52 and tested heterozygous for the alteration. No T/T homozygotes were found among the controls. TopBP1 protein levels were assessed in two wild-type and four Arg309Cys heterozygous LCLs by Western blot analysis. The results showed that heterozygous cell lines did not display any differences in protein levels compared to controls, nor were any aberrant-sized protein products seen (data not shown).

The previously known *TopBP1* alterations in exons 14, 18, 19, 25 and 27 were predicted to have effect on ESE motifs. However, these and the other exonic variants reported in the SNP database, as well as all of the observed intronic changes, were seen at similar frequencies in both cases and controls, and none of them had effect on splice site consensus sequences. Consequently, apart from Arg309Cys, none of the other observed *TopBP1* variants appeared to relate to cancer susceptibility.

4. Discussion

A significant fraction of familial breast and/or ovarian cancer cannot be explained by mutations in known susceptibility genes. TopBP1 encodes a checkpoint protein that is required for DNA damage and replication processes and has both structural and functional similarities with BRCA1. Therefore, to uncover additional predisposing genes we have here performed a comprehensive screening for germline alterations in the TopBP1 gene. Analysis revealed nineteen variants altogether. The novel Arg309Cys substitution, which was observed at significantly higher frequency in familial breast and/or ovarian

cancer patients compared to healthy controls, suggesting about a 2.4-fold increased disease risk for carriers, was the only putative pathogenic alteration found in this study. The variant was not present in ten index cases from *BRCA1/BRCA2* mutation-positive families, but due to the low number of samples tested a possible co-existence of these changes cannot be ruled out. Furthermore, the prevalence of the Arg309Cys variant did not appear to be elevated among breast cancer patients without known family history of the disease.

TopBP1 maps to chromosome 3q22.1 and encodes a 1522 amino acid protein comprising 29 exons.[13,14] None of the observed exon region variants resulted in translational frameshift or nonsense codons, and all missense variants located outside of the BRCT domains. Although the novel missense alteration, Arg1013Gln, as well as the previously known alterations in exons 14, 18, 19, 25 and 27 were shown to have effect on ESE motifs as predicted by the ESEfinder program, equal frequencies in cases and controls indicated that they are likely to be harmless polymorphisms. On the other hand, the novel Arg309Cys alteration caused by C > T transition at codon 1010 was located within a consensus splicing sequence and predicted to affect the consensus acceptor site. However, comparison of TopBP1 protein levels in wild-type and Arg309Cys heterozygous lymphoblastoid cell lines showed normal TopBP1 expression also in Arg309Cys carriers (data not shown), indicating that the variant allele has no effect on TopBP1 protein levels. Neither did the Western blot analysis show any aberrant-sized protein products. These observations imply that the variant would not have any effect at the mRNA level either.

At the protein level, comparison between several species showed that the Arg309 residue is conserved both in dog and frog, and also falls within a region that shows conservation in TopBP1 homologues from yeast to human. In fact, the replacement by a cysteine residue could have harmful impact on polypeptide structure and function, since arginine and cysteine are biochemically profoundly different. Both amino acids are hydrophilic, but while arginine has a guanidinium group as its side chain and is positively charged, cysteine is uncharged and carries a very reactive sulfhydryl group. Arginine at position 309 locates to the N-terminal part of the protein, adjacent to the second BRCT domain (Fig. 1), and some lines of evidence suggest that the amino terminal region of TopBP1 is responsible for transcriptional co-activation functions. The activity of human papilloma virus (HPV) transcription/replication factor E2 was shown to be enhanced by TopBP1, and removal of the amino terminal portion of TopBP1 abolishes this function.[29] Previously the amino terminus of TopBP1 was shown to activate transcription in yeast.[14]

Cell cycle checkpoints are essential for maintaining genomic integrity as they block cell cycle progression in response to DNA damage and allow time for repair processes. The significance of checkpoint defects to human health is illustrated by a growing list of involved genes, such as *BRCA1*, *TP53* and *CHK2* that are mutated in cancer and cancer predisposing syndromes.[3,6,9] TopBP1 participates in recruiting key elements of the checkpoint signalling machinery for cell cycle arrest and DNA damage repair. In addition, TopBP1 displays many similar functions with BRCA1. A recent study reported that both BRCA1 and TopBP1 have a role in propagating signals from ATR to downstream substrates, as well as in mediating interactions between the checkpoint pathway and DNA repair and the replication machinery.[30] The two proteins also have overlapping functions in G2/M DNA damage checkpoint to regulate CHK1 kinase, a key substrate of ATR.[21] In fact, activation of CHK1 phosphorylation in response to a stalled replication fork has been shown to be dependent on TopBP1, and this event is needed for the appearance of DNA damage foci.[31] Furthermore, TopBP1 re-localises to replication forks after DNA damage and has been suggested to have an important role in the rescue of stalled forks.[14] The significance of TopBP1 in checkpoint responses and genomic stability is further emphasised by a very recent study which suggests a crucial role for TopBP1 in ATR activation upon induction of ATR-dependent signalling in response to genotoxic stress.[32] The known functions of TopBP1 indicate that it has an important role in cellular growth control and that defects in its function may lead to tumour development.

A significant fraction of familial breast and/or ovarian cancer cannot be explained by mutations in known susceptibility genes. In the present study, we have analysed the possible role of *TopBP1* in breast and/or ovarian cancer predisposition. We identified one novel putative pathogenic coding variant, Arg309Cys, which was found at significantly higher frequency among the familial cancer cases compared to controls. Segregation analysis with limited number of samples from additional family members revealed incomplete segregation of the alteration with the disease phenotype, as both affected non-carriers and healthy carriers were identified. This observation together with the high prevalence of the allele also in healthy controls and breast cancer patients without known family history of the disease implicates that *TopBP1* Arg309Cys is a commonly occurring allele that may have a subtle impact on cancer risk. These findings fit to the recently proposed polygenic model of breast cancer susceptibility, according to which several low-penetrance alleles with multiplicative effects account for a significant portion of the familial clustering of breast or ovarian cancer in the absence of mutations in the *BRCA1* and *BRCA2* (high-risk susceptibility) genes.[11,12] In conclusion, our results suggest that heterozygosity for the commonly occuring *TopBP1* Arg309Cys allele may confer a moderately elevated risk of breast and/or ovarian cancer, perhaps by acting in concert with other predisposing factors. To our knowledge this is the first report of a mutation screening of the *TopBP1* gene and further evaluation of the current findings will be needed.

Conflict of interest statement

None declared.

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