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***BARD1* variants Cys557Ser and Val507Met in breast cancer predisposition**

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***BARD1* (BRCA1-associated RING-domain 1) is a tumor suppressor whose protein product interacts with BRCA1, and in which rare somatic and germline mutations have been reported in breast, uterine, and endometrial cancers. We aimed to evaluate whether there are *BARD1* genetic variants that contribute to breast cancer risk by screening the gene for germline alterations in 45 Finnish familial breast cancer patients and in seven patients with both breast and ovarian cancer. Two of the missense alterations identified (Cys557Ser and Val507Met) were recently suggested to associate with an increased breast cancer risk. We also analyzed these variants in large and independent series of familial and unselected breast cancer patients and healthy controls. No clearly deleterious mutations were detected in the initial mutation screening. No association of the Cys557Ser and breast cancer risk was observed as the variant was found altogether in 1.4% (16/1181) of familial and 2.2% (34/1565) of unselected breast cancer patients, and in 2.5% (27/1083) of healthy controls. The frequency of the Val-allele of the Val507Met variant was modestly higher among breast cancer patients than among healthy controls, although the difference did not reach statistical significance. No statistically significant association of the Cys557Ser or Val507Met variants with any clinicopathologic parameters was observed. These results suggest that the contribution of the *BARD1* germline variants to breast cancer predisposition is very limited, and that neither Cys557Ser nor Val507Met have an effect on familial breast cancer susceptibility.**

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Introduction

Thus far, two highly penetrant breast and ovarian cancer susceptibility genes, *BRCA1* and *BRCA2*, have been identified. Germline mutations in these genes are found in the majority of families (75–80%) with both early-onset breast cancer and ovarian cancer, but in a considerably lower

fraction of families with site-specific female breast cancer (no ovarian cancer or male breast cancer).^{1,2} Recently, the *CHEK2* 1100delC and Ile157Thr have been identified as new low-risk breast cancer susceptibility alleles.^{3–5} It has been suggested that a substantial proportion of the remaining familial aggregation of breast cancer might be attributed to several such low penetrance alleles that act multiplicatively, and thereby form a polygenic basis for breast cancer predisposition.^{6,7}

One potential candidate gene for breast cancer susceptibility is the BRCA1-associated RING-domain 1, *BARD1* (GenBank U76638). The *BARD1* protein was originally identified through a yeast two-hybrid screen using a BRCA1 RING domain as bait.⁸ The two proteins are structurally related: they both have an amino-terminal RING-domain and one (BRCA1) or two (*BARD1*) carboxy-terminal BRCT domains. The RING domain mediates protein–protein interactions and may also participate in ubiquitination of the proteins, whereas the BRCT domain is found in a large number of proteins that predominantly participate in cell cycle checkpoint or DNA-repair pathways in response to DNA damage. These domains are evolutionarily well conserved, whereas the remaining of the sequences in both genes, with the exception of the ankyrin repeats of *BARD1* that also participate in protein–protein interactions, display a markedly lower degree of conservation.

The interaction between BRCA1 and *BARD1* is mediated via their RING-finger motifs.⁸ The interaction is abolished by tumorigenic missense mutations in the RING domain of *BRCA1*, implying that the formation of a stable complex between these proteins may be an essential aspect of BRCA1-mediated tumor suppression.⁸ A germline *BARD1* mutation has also been reported in a patient diagnosed with breast, ovarian, and endometrial cancer, and somatic *BARD1* mutations have been observed in breast and endometrial tumors; this suggests that *BARD1* mutations might themselves be contributing factors in the development of some human carcinomas.⁹ This is further supported by the loss of the wild-type alleles both in the tumor tissue of the aforementioned patient with a germline mutation, as well as in the endometrial tumor with a somatic mutation.⁹ Functional analysis of the protein with a tumor-associated *BARD1* germline mutation, Gln564His, has also shown that it is defective in p53-mediated apoptosis induction and results in reduced binding to polyadenylation factor CstF-50.^{10,11} Reduced *BARD1* expression has also been observed in some sporadic breast tumors.¹²

As *BARD1* is a plausible breast cancer susceptibility gene, we aimed to evaluate whether there are *BARD1* genetic variants that contribute to breast cancer risk by screening the *BARD1* gene for germline alterations among 45 Finnish *BRCA1/2* negative breast cancer families as well as in seven patients diagnosed with both breast and ovarian cancer.

During the course of this study, two of the identified *BARD1* variants, Cys557Ser and Val507Met, were suggested to associate with breast cancer risk. Cys557Ser was suggested to increase familial breast cancer risk by four-fold,¹³ and the Val507Met was associated with a two-fold elevation in postmenopausal breast cancer risk.¹⁴ We evaluated these variants in extensive series of unselected and familial breast cancer patients, and healthy controls. We also investigated the possible association of the variants with breast tumor characteristics, age at cancer diagnosis, and survival.

Patients and methods

In the initial mutation analysis, the whole *BARD1* coding region was screened in 45 families who have at least three first- or second-degree relatives, including the proband, affected with breast or ovarian cancer. The recruitment of the families, verification of the cancer diagnoses, and exclusion of *BRCA1* and *BRCA2* mutations have previously been described.^{4,15,16} As a functionally relevant *BARD1* germline mutation was found in a patient with three primary tumors (breast, ovarian, and endometrial⁹), seven more patients with both breast and ovarian cancer were included in the analysis.

The frequency of the *BARD1* Cys557Ser was evaluated in two large, independent series of breast cancer patients and healthy population controls originating from Southern and Central Finland. The first series included 888 unselected (described in detail in Syrjäkoski *et al*¹⁷ and Kilpivaara *et al*¹⁸) and 946 familial¹⁶ breast cancer patients collected at the Helsinki University Central Hospital in Southern Finland. *BRCA1* and *BRCA2* mutations have been excluded in all the 403 patients with a strong family history of cancer (three or more first- or second-degree relatives with breast or ovarian cancer in the family, including the proband) as well as in 291 cases with one affected relative as previously described^{2,15,17} (also unpublished data). DNA from altogether 736 healthy female subjects collected at the same geographical region of Southern Finland was studied as healthy population controls. The second series where the frequency of the *BARD1* Cys557Ser alteration was analyzed consists of 697 unselected breast cancer patients collected in Central Finland at Tampere University Hospital, Tampere, Finland¹⁷ (also unpublished data). Of these, 442 patients have no family members affected with breast or ovarian cancer in addition to the proband, 183 have one, and 72 have two or more. *BRCA1* and *BRCA2* mutations have been excluded in 400 of the patients.¹⁷ Also, 358 healthy population controls from the same geographical region were genotyped for the *BARD1* Cys557Ser alteration.

The frequency of the *BARD1* variant Val507Met was studied in 888 unselected and in 710 familial breast cancer patients as well as in 736 population controls from the

Helsinki series. As the variant has been associated with an elevation in postmenopausal breast cancer risk, the patients as well as the population controls were sub-grouped according to their menopausal status (age 50 years was chosen as a surrogate for menopause: patients diagnosed <50 years were considered as premenopausal, and patients diagnosed ≥50 years as postmenopausal).

Information on tumor characteristics (histology, grade, and size of the tumor; nodal status and distant metastasis; estrogen and progesterone receptor status) and age at breast cancer diagnosis was available from 1296 unselected breast cancer patients (altogether 1340 tumors).^{17,18} Written informed consent was obtained from each participating patient. This study has been performed with appropriate permissions from The Ministry of Social Affairs and Health in Finland as well as The Ethics Committees of the Departments of Obstetrics and Gynecology, and Oncology, in Helsinki University Central Hospital, Helsinki, and in the Tampere University Hospital, Tampere, Finland.

Whole-gene mutation analysis and genotyping

The mutation screening of the whole *BARD1* coding region and exon–intron boundaries was carried out by conformation-sensitive gel electrophoresis (CSGE). The detection was based either on autoradiography, where one of the PCR primers was labeled with [γ -³³P]dATP (Amersham Biosciences, Uppsala, Sweden), or by staining the CSGE gels with silver nitrate (MERCK, Darmstadt, Germany). Primer sequences and PCR conditions used in CSGE analysis are available upon request. Samples with a band shift were reamplified, and the nucleotide changes were verified by direct sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit and ABI 310 Sequencer (Applied Biosystems, Foster City, CA, USA).

The genotyping of DNA samples for the nt 1743 G→C (Cys557Ser) and nt 1592 G→A (Val507Met) variants in the Helsinki patient series and population controls was performed using Amplifluor™ fluorescent genotyping (K-Biosciences, Cambridge, UK). The genotyping for codon 557 was successful in 868/888 (98%), 926/946 (98%), and 725/736 (99%) of the unselected and familial breast cancer

patient and control samples, respectively, and 867/888 (98%), 663/710 (93%), and 718/736 (98%) of the studied samples were successfully analyzed for codon 507, respectively. The genotypes of Tampere patients and healthy controls were determined by minisequencing.¹⁹

Statistical analyses

Associations between the *BARD1* genetic alterations and breast cancer risk, as well as the tumor characteristics of the breast tumors, were tested by the χ^2 test. The statistical analyses were carried out in the SPSS software (version 12.0 for Windows, SPSS, Chicago, IL, USA). All *P*-values were two-sided, and *P*-value <0.01 was considered statistically significant as suggested for the association studies for potential low-penetrance alleles.²⁰

Results and discussion

Screening of the *BARD1* gene for germline variants revealed seven coding sequence alterations (Table 1). The rare variants 1145del21 and Arg658Cys found in one family each (1.9%) were also detected in 0.8% (8/974) and 1.9% (18/975) of healthy population controls, respectively (data not shown). The heterozygote carrier frequency of Arg378Ser was also similar in familial breast cancer patients (40.4%, 21/52) and healthy population controls (39.8%, 97/244). These, as well as the silent variants Thr351Thr and His506His, have been described previously, and classified as polymorphisms with likely having no effect on cancer risk.^{9,13,14} Comprehensive analyses were carried out on the identified variants Cys557Ser and Val507Met that were recently suggested to associate with an increased breast cancer risk. These are described in more detail in the following.

The *BARD1* cysteine 557 is a conserved amino acid that resides between the ankyrin repeats and the BRCT domain of a protein. This region has been shown to be essential for apoptosis induction and the interaction of BARD1 with a proto-oncogene Bcl-3.^{21,22} The Cys557Ser alteration was recently suggested as a familial breast cancer allele in breast cancer families from Northern Finland.¹³ We found the variant here in one of the familial patients screened in the

Table 1 Observed coding region variants of the *BARD1* gene

Exon	Nt change	Effect on protein	Frequency for heterozygotes	Previously reported in reference
4	1126G→C	Thr351Thr	17.3% (9/52)	9,13
4	1145del21	7 aa deletion	1.9% (1/52)	9, 14
4	1207G→C	Arg378Ser	40.4% (21/52)	13, 14, 23
6	1591C→T	His506His	7.7% (4/52)	13, 14
6	1592G→A	Val507Met	50% (26/52)	9, 13, 14
7	1743G→C	Cys557Ser	1.9% (1/52)	9, 13
10	2045C→T	Arg658Cys	1.9% (1/52)	9, 13

Nt = nucleotide.

Table 2 BARD1 Cys557Ser frequencies among two independent breast cancer study cohorts and healthy controls

Study cohorts and subgroups	Helsinki series						Tampere series					
	With Cys557Ser						With Cys557Ser					
	Total (n)	(n)	%	OR ^a	95% CI	P	Total (n)	(n)	%	OR ^a	95% CI	P
Population controls	725	19	2.5				358	8	2.2			
Unselected breast cancer patients	868	17	2.0	0.74	0.38–1.44	0.376	697	17	2.4	1.09	0.47–2.56	0.836
With no family history of breast or ovarian cancer	636	12	1.9	0.71	0.34–1.48	0.365	442	13	2.9	1.33	0.54–3.23	0.534
Familial breast cancer patients	926	12	1.3	0.49	0.24–1.01	0.049	255	4	1.6	0.70	0.21–2.34	0.557
By cancer type(s)												
Breast cancer only	797	11	1.4	0.52	0.25–1.10	0.082	222	4	1.8	0.80	0.24–2.70	0.722
Including ovarian cancer	129	1	0.8	0.29	0.04–2.19	0.202	33	0	0			
By no. of affected relatives												
Two affected first-degree relatives	523	6	1.1	0.43	0.17–1.09	0.067	183	3	1.6	0.73	0.19–2.78	0.643
Three or more affected family members	403	6	1.5	0.56	0.22–1.42	0.216	72	1	1.4	0.62	0.08–5.00	0.647

^aCompared to population controls.

initial analysis, and further studied it in two independent series of breast cancer patients and population controls. In the first study series, the Cys557Ser variant was observed with a low frequency both among unselected (2.0%, 17/868) and familial (1.3%, 12/926) breast cancer patients. It was also present among healthy population controls with a frequency of 2.5% (19/725) (Table 2). In the second independent series, the results were similar, with a frequencies of 2.4% (13/442) among unselected and 1.6% (4/255) among familial breast cancer patients, and 2.2% (8/358) among healthy controls (Table 2). Breast cancer patients were further divided into subgroups based on the number of affected relatives and whether or not there were ovarian cancer patients in the family (Table 2). No evidence for elevated breast cancer risk of the BARD1 Cys557Ser variant was observed in any of the study cohorts or subgroups; indeed, the variant frequency was slightly lower among the familial patient series than among population controls. No association between the variant and any of the tumor characteristics (tumor histology, grade, and size; nodal status and distant metastasis; estrogen and progesterone receptor status) was seen either (data not shown), and the mean age at breast cancer diagnosis was similar between the carriers and non-carriers of the variant (57.3 and 58.2 years, respectively ($P = 0.723$)).

Previously, the Cys557Ser variant has been observed in two sporadic Caucasian breast/uterine/ovarian tumors from the US population,⁹ and in one Italian breast/ovarian cancer family.²³ In the former study, the variant was classified as a polymorphism and in the latter, possibly cancer related. Association analysis to evaluate breast cancer risk was not possible in either study. In another recent study, the variant was found in seven out of 126 (5.6%) breast cancer families from Northern Finland and in 14 out of 1018 (1.4%) healthy population controls, suggesting that the carriers would have a four-fold increased risk for familial breast cancer.¹³ The prevalence was highest among patients whose family history included only breast and not ovarian cancer (7/94, 7.4% vs 14/1018, 1.4%; OR 5.8; 95% CI 2.3–14.7, $P = 0.001$). Our results with two extensive and independent series of breast cancer patients do not support association of Cys557Ser with breast cancer risk either among familial or unselected breast cancer patients. We found the variant altogether in 1.4% of 1181 familial breast cancer patients, in 2.2% of 1565 unselected breast cancer patients, and in 2.5% of 1083 healthy controls, respectively, and there was no difference in the frequencies by different family history. The discrepancy between the present study and the study by Karppinen *et al*¹³ (2004) could be due to the small sample size in the latter, which may have resulted in a positive association by chance. Differences in the population stratification of the breast cancer families and population controls used could also be a possible cause

Table 3 Genotypes of the *BARD1* Val507Met in 867 unselected and 663 familial breast cancer patients, and in 718 healthy controls

	Cases		Controls		OR ^a	95% CI	P
	n	%	n	%			
<i>Unselected breast cancer patients, all (n = 867)</i>							
AA	178	20.5	718	24.9	1		
AG	448	51.7	355	49.4	1.27	0.99–1.63	0.061
GG	241	27.8	184	25.6	1.32	0.99–1.75	0.056
GG+AG	689	79.5	539	75.1	1.29	1.02–1.63	0.037
<i>Unselected breast cancer patients, diagnosis <50 years (n = 276)</i>							
AA	52	18.8	468	25.4	1		
AG	138	50.0	227	48.5	1.39	0.94–2.05	0.095
GG	86	31.5	122	26.1	1.61	1.05–2.47	0.027
GG+AG	224	81.2	349	74.6	1.47	1.02–2.12	0.039
<i>Unselected breast cancer patients, diagnosis ≥50 years (n = 591)</i>							
AA	126	21.7	250	24.0	1		
AG	310	52.4	128	51.2	1.15	0.80–1.67	0.450
GG	155	26.2	62	24.8	1.19	0.78–1.82	0.421
GG+AG	465	79.7	190	76.0	1.17	0.82–1.66	0.392
<i>Familial breast cancer patients (n = 663)</i>							
AA	161	24.3	718	24.9	1		
AG	331	49.9	355	49.4	1.04	0.80–1.34	0.786
GG	171	25.8	184	25.6	1.03	0.77–1.39	0.830
GG+AG	502	75.7	539	75.1	1.04	0.81–1.32	0.780

A codes for Met and G codes for Val.

^aCompared to AA genotype.

for a chance association. However, the possibility that the Cys557Ser variant interacts with another, unknown risk allele in the breast cancer families from Northern Finland, but not in Central or Southern Finland, cannot be excluded.

The Met-allele of the common *BARD1* missense substitution Val507Met has been suggested to associate with an increased breast cancer risk in postmenopausal women in a study including 73 pre- and 70 postmenopausal breast cancer patients and 152 healthy controls (adjusted OR 2.05; 95% CI 1.01–4.16).¹⁴ The residue resides within the conserved ankyrin repeat domain, which is responsible for the protein–protein interactions. The particular codon is, however, not fully conserved.

The *BARD1* Val507Met was found to be very common also in our study population. The allele frequencies for G (coding for Val) and A (coding for Met) were 0.54 and 0.46 among the unselected breast cancer patients, 0.51 and 0.49 among the familial breast cancer patients, and 0.50 and 0.50 among the healthy population controls, respectively. The Val-allele (either in homo- or heterozygous form) was slightly more common among unselected breast cancer patients than among healthy controls (OR 1.29; 95% CI 1.02–1.63; $P=0.037$; Table 3). The difference in frequency was highest for the Val-homozygotes, although the statistical significance was not reached (Table 2). When the patients were subgrouped according to their menopausal status, the higher Val-allele carrier frequency was found

in the pre- (OR 1.47; 95% CI 1.018–2.12; $P=0.039$) but not in the postmenopausal women (OR 1.17; 95% CI 0.82–1.66; $P=0.392$), when compared to Met-homozygotes. Similar to the whole cohort, the difference in frequency was highest for the Val-homozygotes (OR 1.61; 95% CI 1.05–2.47; $P=0.028$). Results from the three-value trend test suggested a slightly elevated cancer risk for the Val-allele carriers in the premenopausal patient group but not in the whole cohort of unselected patients (data not shown). The age at breast cancer diagnosis did not differ between the different genotype carriers (55.8 years for the Met-homozygotes, 56.2 years for the heterozygotes, and 55.9 years for the Val-homozygotes). No association between the Val507Met and any of the tumor characteristics or cumulative 5-year survival among the unselected patients was observed (data not shown). Also, no increase in breast cancer risk was observed in familial breast cancer patients. Altogether, our results suggest that this variant does not have an effect on familial breast cancer susceptibility, whereas some evidence for a modest elevation in breast cancer risk among unselected breast cancer patients, especially among those below 50 years, was obtained.

In summary, rarity or absence of clearly pathologic *BARD1* mutations in this study and previous studies suggests that *BARD1* is not a major gene underlying familial breast cancer. Our results also indicate that neither the *BARD1* Cys557Ser nor Val507Met variants predispose to familial breast cancer.

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